Tumor neo-angiogenesis in locally advanced breast cancer: A clinico-pathological study

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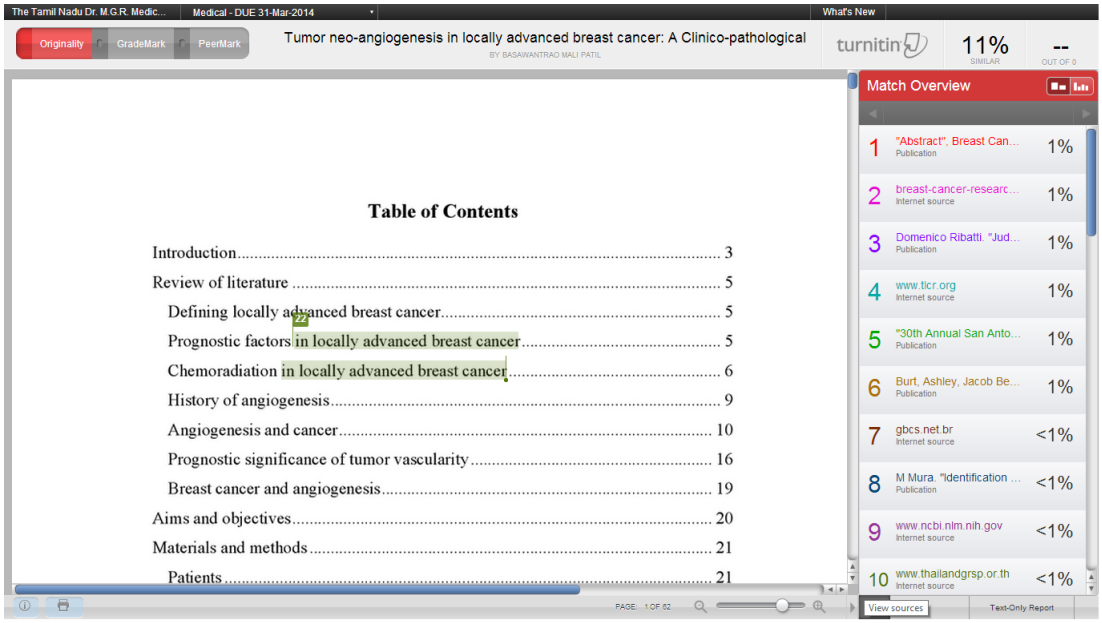
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Certificate

This is to certify that the dissertation entitled **"Tumor neo-angiogenesis in locally advanced breast cancer: A clinico-pathological study"** is a bonafide work done by **Dr. Basawantrao Malipatil**, in the Department of Medical Oncology, College of Oncological sciences, Adyar, Chennai, in partial fulfilment of the University rules and regulations for award of Doctor of Medicine in Medical Oncology under my guidance and supervision during the academic year 2011 to 2014.

Prof. T. S. Ganesan
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Certificate

The Institute Thesis Review Committee (IRTC) reviewed and approved the dissertation entitled "**Tumor neo-angiogenesis in locally advanced breast cancer: A clinico-pathological study**".

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Summary

Breast cancer is among the five most common cancers in India. The incidence of locally advanced breast cancer (LABC) is high because of lack of awareness about health among women. At our center patients with LABC are initially treated with neoadjuvant concurrent chemoradiation before primary surgery.

We studied the role of neoadjuvant chemoradiation in patients with LABC in improving outcomes. Data of 135 consecutive patients who presented with LABC treated in 2007 was analyzed. All these patients received protocol neoadjuvant chemotherapy with concurrent radiation to breast and axilla before primary breast surgery.

Overall, in this cohort of patients' disease free survival (DFS) was 72.5% and overall survival (OS) was 75% at 5 years. Evaluation of all clinical variables and their correlation with outcomes was performed. The prognostic factors that correlated with improved DFS on univariate analysis were older age (P = 0.036), clinical nodal stage 1 (P = 0.008) and pathological complete response in nodes (P = 0.002). Similarly, for OS the factors that correlated on univariate analysis were older age (P = 0.There was a statistically significant improvement in disease free survival with older age (P = 0.009), clinical tumor size (P = 0.025), clinical nodal stage 1 (P = 0.014) and pathological complete response in axillary nodes after surgery (P = <0.001). In multivariate analysis two models with and without clinical and pathological nodal stage was evaluated with other factors that were significant in univariate analysis. The best independent prognostic factors predicting better DFS were clinical nodal stage (P = 0.009) and pathological nodal stage (P = 0.015) and

for OS were age (P = 0.022), clinical nodal stage (P = 0.017) and pathological nodal stage (P = 0.009).

We also evaluated the role of angiogenesis in LABC, by evaluating microvascular density (MVD) using CD31 as a marker for blood vessels. Forty three out of 55 available tissue paraffin blocks were positively expressing CD31. The median MVD of 43 tumors was 52. In view of the small number of samples, there was no correlation with outcome.

Management of locally advanced breast cancer with concurrent chemoradiation is feasible and the long term outcome at 5 years is comparable and may be superior to other approaches. Further evaluation of MVD by different vascular markers is required to assess its importance in LABC.

Chapter 1

Introduction

India with a population of 1.2 billion (2011 census) is the most populous democratic country in the world and will surpass China by 2025 (1). Though the incidence of communicable diseases is still challenging the burden of non-communicable diseases like cancer is increasing in India.

The incidence of breast cancer has increased over the decades around the world. It was estimated that there were 1.5 million cases of breast cancers in 1999 a 82% increase from year 1990 (2). Breast cancer is the most common cause of cancer death among women only next to lung cancer (3).

The greatest increase in incidence has been in Asian women which peaks in the forties in contrast to Western women where it peaks in the sixties (4,5). Fifty percent of Indian women are premenopausal (5) and has been projected that majority of breast cancer burden will be in Asian women.

The incidence of breast cancer in India until about 10 years ago was 10 per 100,000 women which has increased currently to 23 per 100,000 (6). There were 115,000 patients with breast cancer and 53,000 deaths (3) in 2008. The rate of increase in incidence of breast cancer is 0.5 – 2% per annum and more so in younger age groups (<45 years) (7). As the median age of the population of women in India is young, the proportion and median age of patients with breast cancer is also less than 50. For example, the median age of patients with breast cancer in 6 hospital-based cancer registries ranged from 44.2 years in Dibrugarh, 46.8 years in Delhi, 47 years in Jaipur, to 49.6 years in Bangalore and Chennai. The average age reported from other parts of country varies from 50 – 53 years (National Cancer Registry

Programme, 2001) with significant population younger than 35 years (11% at TMH, Mumbai (8), and 26% at SGPGIMS, Lucknow (5)).

The awareness of breast cancer is good in the West and in addition, better screening and health care facilities have improved the survival of patients to >80% in North America and Europe compared to 60% and 40% in middle-income and low-income countries respectively (9). There is even variation in presentation with more women presenting in early stage in the West compared to those with advanced disease in the developing world (10).

69 percent of the patients presenting to our hospital have locally advanced breast cancer (LABC) (11). Understanding the pathogenesis of the disease will allow the development of targeted drugs (including anti-angiogenic therapy) other than chemotherapy to tackle this huge burden.

Review of literature

Defining locally advanced breast cancer

Breast cancer can be grouped clinically into early, locally advanced and metastatic. The early breast cancers are stage I and II of AJCC staging system (12). Stage III breast cancers are considered to be LABC (13). LABC can be further classified into operable (IIIA: T3 with N1; N2 with T1-3) and inoperable (IIIB: T4a, T4b, T4c, T4d; IIIC: N3 with any T) cancers. Metastatic disease is any breast cancer which has spread beyond supraclavicular nodes (13).

Locally advanced breast cancers remains a peculiar group although the disease is locally advanced, many of the patients do not have metastases. The survival of consecutive unselected patients with LABC treated with concurrent chemoradiation and surgery at Cancer Institute at 15 years is 48% (11).

Prognostic factors in locally advanced breast cancer

A variable which helps in the prediction of outcome of disease is called the prognostic factor. Prognostic factors help in better risk stratification of patients with breast and tailoring their management. A number of prognostic factors have been associated with breast cancer; the most important being tumor size, nodal metastasis, histologic subtype, grade of the tumor, estrogen receptor status and the HER-2 expression in the tumor (14,15). Among them the most important prognostic marker is the lymph nodal status. As per United States Surveillance, Epidemiology, and End Results (SEER) database the 5-year survival rates dropped from 92% to 81% and 57% for node-negative, 1-3 nodes and >3 node positive group respectively (16).

Although nodal metastasis in breast cancer remains the most important factor, there are many other attributes in the tumor that determines outcome in a particular patient.

The concerted application of modern tools of molecular biology which include sequencing and expression studies have helped in identifying approaches to and prediction of outcomes in breast cancer e.g., Oncotype Dx (17) and Mammaprint (18). The role of such prognostic markers has also helped in developing newer targeted therapies against breast cancer. Angiogenesis being necessary for growth and metastasis of cancer has been shown to be a prognostic marker in solid tumors including breast cancer.

Chemoradiation in locally advanced breast cancer

The use of neoadjuvant therapy in LABC was developed to reduce the size of the tumor and to bring inoperable tumors in the purview of surgery. This was particularly important when breast conserving surgery was the ultimate goal. In addition neoadjuvant chemotherapy in LABC helps in assessing the pathological response to such treatment thereby improving further adjuvant therapy (19).

Surgery although the mainstay of treatment for patients with inoperable breast cancer, historically chemotherapy or radiation was given as adjunct to bring them under the purview of surgery. The administration of neoadjuvant chemotherapy improved the operability in LABC and the response rates have dramatically improved over the years with achievement of clinical complete responses ranging from 10% to 30% and partial responses of 50-60% (20). Increase in clinical response rates was associated with improved pathological response rates (of about one fifth to one-third) and survival outcomes (21). The largest trial National Surgical Adjuvant

Breast and Bowel Project (NSABP) B-18 trial compared neoadjuvant chemotherapy versus adjuvant doxorubicin plus cyclophosphamide in 1523 women with median breast tumor size of 3.5cms. There was no difference in OS and DFS in both arms (HR = 0.99; 95% CI, 0.85 to 1.16; *P* = .90) and DFS (HR = 0.93; 95% CI, 0.81 to 1.06; *P* = .27) but a trend towards better OS with neoadjuvant therapy in women younger than 50 years was observed (OS: HR = 0.81, *P* = .06; DFS: HR = 0.85, *P* = .09) (22). Pathological complete response (pCR) in neoadjuvant arm was 13% and those who achieved pCR had superior disease free survival (DFS) and overall survival (OS) compared to those not achieving pCR (DFS: HR = 0.47, *P* < .0001; OS: HR = 0.32, *P* < .0001). In the patients who received neoadjuvant chemotherapy only 5% were LABC (cT3N1). The phase III EORTC 10902 trial which included T1c – T4b tumors and nodal stage ranging from N0 – 1 showed a pCR rate of only 4% with FEC based chemotherapy (19). NSABP B-27 trial with addition of docetaxel to AC chemotherapy could increase the pCR to 26% but the meta-analysis of trials of neoadjuvant chemotherapy showed a pCR range of 4% - 29% (23).

Combining radiation with chemotherapy can improve response rates and outcomes in LABC in neoadjuvant setting. Preoperative chemotherapy with AC x 6 cycles followed by radiotherapy (50 Gy plus a 10 Gy boost) could not significantly increase the pCR rate (24). Matuschek et al (25) showed a pCR rate of 29.2% with neoadjuvant chemoradiation and were able to do breast conservation surgery in 50.8% of patients with LABC. With twice a week paclitaxel together with radiation given concurrently (45 Gy at 1.8 Gy/fraction) in a phase I/II trial, 34% of patients with LABC could achieve pCR (26) with better DFS and OS (27).

Table 1: Neoadjuvant chemotherapy trials in breast cancer (22,23,28–31)

Trials	Phase (n)	Tumors	Chemotherapy	Primary end points	pCR rates
Royal Marsden	III (293)	T0–4, N0-1	4 × 2MT	BCT 89 versus 78% (P =.004)	pCR 7%
NSABP B-18	III (1493)	T1–3, N0-1	4 × AC	5 y-OS: 80 versus 81% (ns); 5 y-DFS: 67 versus 67% (ns)	pCR 13%
EORTC 10902	III (698)	T1c–T4b	4 × FEC	4 y-OS 82 versus 84% (P = .38)	pCR 4%; downstaging to BCT in 23%
ABCSG-7	III (423)	T1–3, N0-1 HR− + high risk HR+	3 × CMF	RFS better with adjuvant therapy (HR 0.7; P = .02); no difference in OS (HR 0.8; P = .21)	pCR 6%
Meta-analysis	IV (3946)	9 randomized trials	Same regimen	No difference in OS (RR 1.0); no difference in DFS (RR 0.99)	pCR range 4–29%
NOAH	III (235)	HER2+ LABC	3xAP + 4xP + 4Xec + 3xCMF + H	Improved 3-year EFS (71%)	pCR 43%

Retrospective analysis of patients with breast cancers at our hospital from 1990 to 1999 showed that among 1117 patients With CMF based regimen when used concurrently with radiation the tumor down staging was possible in 45% of 1117

patients and nodal down staging by 57.5% at our hospital (11). In India because of lack of general health awareness most of the women with breast cancer present in advanced stages as reported by our hospital in the above study in which 65% of patients were LABC

History of angiogenesis

It is clear that except during embryonic development, physiological angiogenesis during adult life does not occur. The only exceptions to this rule are the ovary and endometrium where physiological angiogenesis occurs. Normally blood vessels are quiescent and pathological neo-angiogenesis is prerequisite for growth of tumors. Evidence that tumor releases specific factors for formation of new blood vessels was shown in 1939 by Ide et al (32). In 1945, Algire and Chalkley (33) could show that angiogenic response was more substantial and earlier in tumor than normal tissues when implanted in cat's skin. They found that tumor growth was linked to a developing vascular network. In 1956, Melwin and Algire (34) demonstrated that vaso-proliferative response induced by tumor penetrated the tissue in which it was implanted. The distance between implant and host's tissue with normal vessels if beyond 50μm was unable to induce any kind of response. However tumor tissue could. In 1968, Greenblatt and Shubik (35) using Millipore chambers implanted in hamsters cheek which was not permeable to tumor cells could demonstrate new blood vessel formation with growing tumor was possible by probable release of diffusible factor that could pass through the pores. These data were confirmed in chick embryo chorioallantoic membrane using millpore filters tumor fragments laid on it (36).

In 1963, Folkman and Becker demonstrated that tumor cells implanted into isolated organs could not grow beyond 1 – 2 mm diameter and also could not get vascularized in presence of free hemoglobin solutions lacking platelets (37). These tumor cells when reimplanted into host mice became vascularized and grew beyond 1 – 2 mm^3 (38). This was the first evidence showing that neovascularization is prerequisite for growth of tumor. Folkman in 1971 was the first to hypothesize that tumor growth is dependent on angiogenesis and that "anti-angiogenesis" could be therapeutic by preventing formation of new vessel sprout (39). Folkman also said that for tumor cannot sustain growth beyond 1-2 mm^2 size without formation of new capillary blood vessels. Neovascularization is necessary for providing oxygen and nutrients to the dividing tumor cells.

Judah Folkman is now considered as father of angiogenesis research (40) and his concepts have now been widely accepted in this field over the years.

Angiogenesis and cancer

Angiogenesis is required for the growth and maintenance of the tumor (39). It is a process of formation of new blood vessels from pre-existing blood vessels (41). Tumor angiogenesis is proliferation of blood vessels to support the increasing demand for oxygen and nutrients by the growing tumor tissue. Angiogenesis can be either by vasculogenic mimicry (tumor cell differentiation into endothelial-like cells) or by angiogenic stimulus by tumor cells (42). Many subtypes of angiogenesis have been described. They are described below.

a) Sprouting angiogenesis is proliferation of endothelial cells and formation of solid spurts with extension towards angiogenic stimulus (43,44),

Steps involved in sprouting angiogenesis are (45):

i. Formation of a capillary sprout from the pre-existing mature blood vessel after degradation of basement surrounding the parental postcapillary venule. The degradation of basement membrane is thought to be by the various pro-angiogenic factors released by the tumor cells including proteolytic enzymes like matrix metalloproteinases, cathepsins and urokinase plasminogen activator. The capillary sprout is from the differentiated endothelial cell which moves towards the angiogenic stimulus secreted by tumor cells
ii. The sprouts migrate towards the angiogenic stimulus, followed by their division thus lengthening the "stalk"
iii. Formation of lumen after the basement membrane and perivascular supportive tissue including pericytes are formed.

The capillary sprouts can be divided into three zones: "tip cells" at growing end, which are non-dividing and specialized to migrate towards angiogenic stimulus and fuse with other tip cells to form capillary network; intermediate zone which has dividing endothelial cells which lengthen the stalk; and the basal endothelial cell zone, which are differentiated to form the endothelium with lumen formation.

(b) Splitting angiogenesis (Intussusception) is division of the lumen of an pre-existing vessel by formation of transvascular tissue pillars which extend into lumen splitting the vessel and resulting in formation two vessels (42,46). It is an alternative and rapid mechanism of new vessel formation with increase in capillary density

within minutes (47) without significant increase in endothelial cell number. It mainly occurs in developing embryo but also perhaps in tumors (48). The pathogenesis of intussusceptive angiogenesis has not been explored largely and can be a probable area for research to find newer ways of treatment for cancer.

(c) Vasculogenesis is formation of vasculature from endothelial stem cells (also called angioblasts), which proliferate into de-novo endothelial cells (49). The angioblasts are committed to vascular lineage and they differentiate to form immature vascular plexus. Vasculogenesis plays a main role in angiogenesis in embryo (50). The origin of endothelial cells has long been debated. Their origin from a common stem cell called hemangiobast has been hypothesised which also form hematopoietic cells but unequivocal evidence is still lacking (51,52). Origin of postnatal vasculogenesis and tumor angiogenesis is still controversial.

(d) Glomeruloid angiogenesis has also been observed in invasive breast cancer where the capillary network form highly complex vascular aggregates resembling glomerulus of kidney called glomeruloid bodies (53). It has been associated with poor outcomes (54). VEGF has been implicated in its formation and has also been inferred to represent vascular remodelling rather than sprouting angiogenesis (55,56).

(e) Vascular mimicry is a neovascularization strategy in which tumor cells replace endothelial cells as cell lining the capillaries. They will also acquire the phenotypic characteristics of an endothelial cells (57,58). Such kind of vascular mimicry by tumor cells have been seen in ocular melanoma and ovarian cancer (59). Few instances of breast tumors where vascular mimicry occurs has also been reported

(60). It is important to recognize this entity as conventional angiogenic inhibitors may not play a role in the treatment.

In tumors, because of chronic overproduction of pro-angiogenic factors causes uncontrolled development of new blood vessels with concomitant increase in number of vessels per unit volume (i.e., microvessel density). The flow of blood in these vessels is both spatially and temporally heterogeneous and, sometimes oscillating in antegrade to retrograde direction.

Normal vascular endothelium has uniform single layer of endothelial cells which have few cytoplasmic projections. Tumor endothelial cells (TECs) are described as having irregular shape and size with ruffled margins, long and fragile cytoplasmic projections which extend outwards, across vessel lumen. Intercellular gaps are created by the tips of these cytoplasmic projections which penetrate lumen creating openings or small intercellular gaps in the vessel wall. These gaps increase the permeability of the vessels many fold. CD31 uptake on immunohistochemistry is spotty which gives “mosaic” appearance of the endothelium (61). This is because of either lack of CD31 expression by TEC or total absence of TEC in the vessel wall with occasional tumor cells expressing VE-cadherin fill the gaps in vessel wall (62) masquerade as endothelium (63), but this remains controversial (58).

Tumor cells enter the circulation by these vessels for hematogenous metastases and have been shown that with the presence of more immature vessels the risk of metastases increases (64). Anti-angiogenic factors were shown to inhibit such metastases. Angiostatin which inhibits angiogenesis was demonstrated in mouse models that if present at higher concentrations will lead to decreased metastasis

(65,66). Decreased VEGF levels in tumor have been associated with defective angiogenesis and inhibition of metastasis (67).

Angiogenesis is restricted to wound healing in normal conditions and is found to be sustained in certain pathological conditions like rheumatoid arthritis, psoriasis and diabetes (68). In experimental models transplanted breast tumor tissues were found to be more angiogenic about 30% compared to normal breast tissue of about 3% (69–71). Angiogenesis being rate limiting for tumor growth as described above, breast tumor in order to sustain growth undergoes angiogenic switch before any morphological changes are identifiable (72). As many as 2000 oncogenes and tumor suppressor genes expression has been found to be altered in this angiogenic switch including Ras, myc, raf, c-erbB-2, c-jun and src (73–77).

The new tumor vessels that formed are poorly organized and leaky with intermittent and sometimes reverse flow(78). This leads to tumor hypoxia and microenvironmental stresses with increased tumor clones resistant to treatment. In presence of low oxygen tension, hypoxia-inducible factor (HIF) is stabilized (hydroxylation of HIF inhibition) with translocation to nucleus. With binding to HIF-1β and other hypoxia response elements to activate several genes involved in angiogenesis, glycolysis, erythropoiesis and apoptosis (79). Also HIF-1α is overexpressed in tumors and has been associated with advanced disease and poorer prognosis (80–82) but its role in breast cancer is not known because of limited data. HIF-2α overexpression has been seen in both tumor cells and in tumor-associated macrophages (83,84) with positive association with increased tumor vascularity(84). Hence HIF has been found to help in tumor vascular remodelling under hypoxic

stress (79). Considering the significant role of HIF in tumor angiogenesis and growth significant research is being performed in targeting this pathway (85,86).

The present research into tumor neo-angiogenesis has also been focusing on the modifying factors of tumor microenvironment. Tumor microenvironment is different in many aspects as described above because of tissue hypoperfusion by disorganized immature vessels and consequent low oxygen tension and poor nutrient supply, acidic extracellular fluid pH, increased interstitial pressure and increased pro-angiogenic factors (87,88). As described above this leads to change in expression of at least 2000 genes. These will lead to expression of different genes and proteins in tumor endothelial cells and targeting against these genes or proteins can help in producing selective drugs targeting the tumor. Using newer methods like serial analysis of gene expression (SAGE), microarray platforms, proteomic analysis, and bioinformatics data mining different new promising tumor endothelial markers have been identified like extra domain B of fibronectin, a series of numbered tumor endothelial markers (TEMs), annexin A and ROBO4 (89,90). Only a few targets against them have even been successful in animal models (91–93) but it has not been shown to be significant in humans breast cancer probably because of more complex regulation of angiogenesis and lack of widespread expression of these targets as was expected (89).

Prognostic significance of tumor vascularity

Use of tumor vasculature and its correlation to prognosticate cancer related outcomes was tried as early as in 1972 by Brem in the Folkman laboratory who correlated histological grade of brain tumors with neovascularization (94). In 1991 Weidner et

al (95) was first to describe the enumeration of microvascular density by immunohistochemistry (IHC). Using specific vascular IHC markers, tissue sections were identified for areas with increased vasculature (hot spots) under low power magnification and then individual vessels were counted in at least 5 such "hotspots" under high power magnification. Their average was taken as microvascular density (MVD). The most commonly used IHC markers for assessment of vascular density are CD31, CD34, and von Willebrand factor (96). To minimize the subjective errors in counting vessels by MVD method a computer based method was devised called Chalkley count in which a 25-point graticule is used for counting hot spots by orienting the points over the area of maximum hotspots (Chalkley grid area: 0.196 mm2) (97). It provided a better but only relative estimate of the vascular density. It is more reproducible minimizing the subjective bias.

Over the years MVD counting protocols described above has been used in many studies and has become the standard for evaluation of tumor angiogenesis. MVD has been significantly associated with prognosis and outcome in various cancers because tumor growth and metastasis are angiogenesis dependent (98–101). Its importance is even more in early stage cancers which tend to relapse with failure of available prognostic parameters e.g., in hepatocellular carcinoma of size <5cms, MVD was shown to be independent prognostic marker (102). Tumor angiogenesis is also considered as prognostic factor in hematological malignancies. Various studies have positively correlated adverse prognosis with increase in bone marrow angiogenesis (103,104).

Though MVD has been shown to be a prognostic factor, a few studies (100,105) could not prove its positive prognostic value. Many reasons have quoted for these negative results. Lack of standardization and objective method of assessment of MVD, counting of microvessel in area of hot spots under low and subsequent high power field leading to subjective bias and inter-observer variation, lack of standardized tissue sampling sites for the tumor subtypes in retrospective studies, in which mostly archived tumor specimens may be used etc. The use of different endothelial markers in different studies is may also have contributed to the variation in results.

Use of computerized or automated method for analyzing the MVD can increase the accuracy of future studies as was shown by as shown by Acenero et al (106) where automated computer based image analysis of MVD was prognostic indicator but not manual counting. Also use of more tumor endothelial specific antibodies can increase the prognostic significance like integrin αvβ3 (107). VEGF overexpression has been found in various studies to be associated with high MVD, advanced stage of tumor (108–111) and in few studies was found to be an independent prognostic factor along with conventional parameters (108,110,111). Overexpression of other angiogenic markers like PD-ECGF (112–114), bFGF (115,116), transforming growth factor (TGF)-β (117–119), angiogenin (120,121), tissue factor (122), and COX-2 (123,124) have also been found to be associated with poor prognosis in various studies. Evaluation of overexpression of such angiogenic factors may be an alternate to overcome the disadvantages associated with use of tumor MVD

Since many of the angiogenic factors are soluble and diffusible peptides, measurement of their levels in body fluids can provide an alternative means to assess the tumor activity. High serum and urine bFGF has been found to be associated with progressive cancers (125). Serum VEGF levels has been more extensively studied and appears promising (126–130). It has been used for response assessment to perioperative or post neoadjuvant therapy (131–134) and to antiangiogenic therapy targeting VEGF (135). The role of soluble VEGF receptor, and ratio between VEGF and soluble VEGF receptor have also been studied (136–138). Correlation between serum VEGF per platelet to tumor activity has also been studied in hepatocellular cancer (139) considering the fact that circulating platelets can also be source of VEGF for which the reliability as a marker has been debated (140).

Studies have also tried to correlate between tumor angiogenesis and response to therapy (chemotherapy and radiotherapy). Dirix et al (1997) was the first to show the higher levels of angiogenic factors VEGF and bFGF to be associated with progressive disease on chemotherapy. Hyodo et al (141) correlated low VEGF levels with responsiveness to chemotherapy in metastatic gastric and colorectal with lower levels of VEGF which was not observed with CEA and CA-19.9. Similar results were observed with patients undergoing chemoradiation for esophageal squamous cell carcinoma (142).

Although the above studies are promising still there are large lacunae in using these markers as the studies have not been entirely consistent in predicting and prognosticating cancers. Also because of substantial overlap of levels with healthy volunteers and wide variation in levels of these angiogenic markers successful

translations to clinical use has not been possible (96). But considering the scope of targeted therapies against tumor angiogenesis there is an unmet need for newer specific markers for tumor angiogenesis.

Breast cancer and angiogenesis

Various studies have shown the role of angiogenesis in pathogenesis of development of breast cancer and its aggressiveness. As the vascular density increases the risk for development of carcinoma breast increases in fibrocystic disease (143). Many angiogenic factors have been implicated in the angiogenesis of breast cancer and VEGF (144) has been shown to be the key pro-angiogenic factor. Patients with C936T polymorphism in VEGF are protected against breast cancer (145). Increased VEGF expression and increased microvascular density have been shown to be associated with aggressive ductal in-situ carcinoma and metastases in women with breast cancer (95,146). Increased VEGF is associated with poorer survival outcomes even irrespective of nodal status (147) and poorer response to treatment (148) (chemotherapy or tamoxifen).

Aims and objectives

1. To evaluate prognostic factors in a cohort of patients treated for LABC in year 2007.
2. To evaluate the significance of prognostic factors and correlate it with outcome in women with breast cancer.
3. To correlate expression of CD31 with clinical and pathological parameters before and after treatment by univariate and multivariate analysis
4. To correlate expression of CD31 with outcome i.e. disease free survival and overall survival.

Chapter 2

Chapter 2: Materials and methods

Patients

From January 2007 to December 2007 consecutive patients with non-metastatic, locally advanced carcinoma of the breast (LABC) will be selected for the study (as they will be having a minimum period of follow up of 5 years).

Locally advanced breast cancer included stage III (A, B, and C). Those patients who were found to have early breast cancer or metastatic disease were not included in the study.

Patients with LABC have been treated with the institute protocol of neo-adjuvant treatment (chemoradiation) prior to definitive surgery (modified radical mastectomy or breast conservation surgery). The neoadjuvant chemoradiation protocol included neoadjuvant chemotherapy of four to six cycles' chemotherapy concurrent with external beam radiation (EBRT).

The protocol chemotherapy which was given q3 weekly included either of following of:

- FAC: 5-Fluorouracil 600mg/m^2, Adriamycin 60mg/m^2, Cyclophosphamide 600mg/m^2
- FEC: 5-Fluorouracil 600mg/m^2, Epirubicin 60mg/m^2 and Cyclophosphamide 600mg/m^2
- CMF: Cyclophosphamide 600mg/m^2, Methotrexate 50mg/m^2, 5-Fluorouracil 600mg/m^2
- TE: Paclitaxel 175mg/m^2 and Epirubicin 60mg/m^2

The protocol for concurrent EBRT fields included whole of breast, axilla and internal mammary region with the total dose of radiation of 41.5Gy (180cGy in 21 fractions). EBRT field also included supraclavicular region if patient had clinical N2 disease or stage IIIA with high risk features like younger age, hormone receptor negative and high grade tumors. EBRT was started with 1st cycle of chemotherapy in patients who had cT3N1 disease and among patients with cT4bN0-2 who didn't have gross breast tissue infection or skin ulceration. Patients with skin ulceration or gross breast tissue infection received few cycles of chemotherapy and radiation was added when satisfactory reduction in tumor size was documented with physicians' assessment of tolerance to concurrent radiation.

At the completion of chemoradiation patients underwent mastectomy with axillary dissection followed by radiation to internal mammary region with dose of 40 - 45Gy if not included during neoadjuvant radiation therapy to patients with inner quadrant tumors, level III node positivity or >3 node positivity post-surgery.

The diagnosis was established in all patients by fine needle aspirate and trucut biopsy from the tumor. The tumor tissue from trucut biopsies of these patients were collected from tumor bank for the present study purpose. All patients subsequently underwent definitive surgery that included mastectomy. Subsequent to the mastectomy patients received further chemotherapy to a total of 6 cycles followed by if appropriate hormonal therapy i.e., tamoxifen

Material

The above mentioned group of patients with LABC was followed up to 31st December 2013 so as to get a follow up period of 6 years (1st January 2008 to 31st December 2013). Data on all patients that included clinical and pathological features including expression of hormonal receptors in the tumor were collected. Analysis of clinical features of all patients was performed to assess (by univariate and multivariate analysis) prognostic factors that correlate with outcome. Outcome measures were defined as pathological response to initial treatment, disease free and overall survival.

Overall survival was defined as duration from date of diagnosis to last follow-up or death and disease free survival was defined as duration from date of diagnosis to date of relapse or death.

Methodology of immunohistochemistry

Paraffin blocks from patients with adequate malignant cells (>70%) were selected for further analysis. The antibody used in the study was specific for CD31. Immunohistochemistry was performed on the sections from the paraffin blocks as follows.

Formalin fixed paraffin embedded tissues were cut into sections of 4 µm thickness and fixed onto glass slides by drying. The tissue sections were mounted on coated slides and dried at 60°C overnight. The sections were deparaffinised with three changes of xylene and then rehydrated in descending alcohol series of 100%, 90% and 80%. The CD31 antigen was retrieved by Heat Induced Epitope Retrieval (HIER) at 95 °C in water bath with citrate buffer (pH 6) for 10 minutes. The tissues

were blocked with Hydrogen peroxide (H_2O_2) as well as blocking solution (Biogenex) to avoid nonspecific binding. The primary antibody used was undiluted mouse anti-human monoclonal antibody, CD31 (Biogenex). The sections were incubated overnight at 4°C. The tissues were further incubated with undiluted secondary antibody (Biogenex) for CD31, conjugated with Horse radish Peroxidase, for 1 hour at room temperature. The chromogen used was diaminobenzidine (DAB). The tissue sections were then counter stained with hematoxylin. The slides were dehydrated with xylene and mounted with a mixture of Distyrene, a plasticizer, and xylene (DPX).

Evaluation of CD31 expression

We could not use the Chalkley method (149) for evaluation of microvascular density as the tumor tissue was too small for counting the hot spots. Hence we counted the entire vessels positive for CD31 at 20X magnification. The vessels were counted by two independent individuals and the average of both the readings was considered.

Statistical analysis

The relationship between the prognostic factors considered (age, clinical tumor size, clinical nodal stage, grade, hormonal status, type of chemotherapy, pathological response and CD31 blood vessel expression) and outcome measures (overall survival and disease-free survival) was evaluated by Cox proportional hazards model using the statistical software SPSS v.22.

The pathological complete response is defined as absence of invasive cancer in both the breast and nodes after surgery (150). The overall survival was defined as duration in months from date of diagnosis to date of last follow-up or death and

disease free survival was defined as duration in months from date of diagnosis to date of relapse or death. Univariate analyses of all these prognostic factors are also presented. To assess the prognostic information by multivariate analysis because of dependency of clinical and pathological nodal status we have split them into two separate models for analysis which included age and pathological complete responses. We have also presented the survival curves using the method of Kaplan and Meier (151).

Chapter 3

Chapter 3: Results

Patient characteristics (Table 2 & 3):

A total of 753 women with carcinoma breast visited to Cancer Institute (WIA), Adyar during the study period. Among them 237 patients were found to have locally advanced breast cancer. In the present study we included 135 evaluable patients who received protocol neoadjuvant chemoradiation.

A total of 102 patients were excluded from study because of various reasons: 12 patients were excluded because they defaulted after neoadjuvant chemoradiation or refused further treatment; 31 patients were excluded because of their tissue diagnosis was established by fine needle aspiration or underwent excision biopsy before coming to our hospital and hence didn't undergo MRM after chemoradiation; 10 patients were excluded because they defaulted after diagnosis or after start of neoadjuvant chemoradiation; 49 patients data was inadequate because of default due to various reasons mentioned above..

These 135 patients have been included in the study as they all had underwent upfront core needle biopsy followed by neoadjuvant chemoradiation and then surgery on breast.

Median age of these patients was 47 (range 22 - 70) (Figure 1). There were 98 patients with clinical stage IIIA and 37 were with clinical stage IIIB but none of these evaluable patients had stage IIIC. The clinical tumor stage ranged from T2 to T4b. There were 9 patients with cT2N2 disease; 89 patients were cT3 and 37patients were cT4b. Ninety six patients (71%) were with cN1 and 39 (29%) were cN2 disease. Among the histological subtypes 126 (93%) patients were having infiltrating

ductal carcinoma and 3 each had mucinous, medullary and other subtypes (Figure 3). Two patients had grade 1 tumors, 60 were grade 2 tumors and 66 were grade 3 tumors. For 7 patients the information on grade of tumor was not available. For statistical analysis these grade 1 & 2 tumors were grouped as low grade and grade 3 has high grade. Patients having ER positive tumors were 75 (55.5%) and patients with ER negative tumors were 58 (42.2%) (For two patients ER status was not available, Figure 2). Similarly there were 65 (50%) patients with PR positive tumors and 65 (50%) with PR negative (PR status for 5 patients was not available). Evaluation of c-erb2 receptor was not routinely performed on tumors during 2007 and therefore not available.

Eighty nine patients (66%) received FAC chemotherapy, 22 (16.2%) FEC-60 and 3 (2.2%) patients received Paclitaxel + Epirubicin chemotherapy. Twenty one patients (15.5%) received CMF chemotherapy (Figure 4). For statistical analysis all these patients have been combined in subgroup receiving anthracycline based chemotherapy. For 119 (88%) patients concurrent radiation was started with first cycle of chemotherapy and rest 16 patients received radiation in subsequent cycles. Among the 135 patients 125 (92.5%) patients underwent Auchincloss type of modified radical mastectomy, 8 patients underwent Patey's mastectomy and one patient underwent segmental mastectomy (breast conservation surgery). One patient progressed on chemoradiation and hence surgery on breast was not offered and this patient subsequently died of disease. Median number of nodes removed during modified radical mastectomy was 11 (range 3 – 25). Median number of nodes positive among those who didn't attain pCR was 2 (range 1 – 11). Forty seven of 134

patients after neoadjuvant chemoradiation had pathological complete response on examination of their tumor and nodes at surgery.

Survival analysis:

The median follow up of the entire cohort of patients was 72 months. In the cohort of 135 patients, 36 relapsed, of which 31 patients have died. Overall 101 patients were alive and 33 were dead at the conclusion of study period. 4 patients who had relapsed during the study period are still alive and doing well. At a median follow-up of 72 months, the median DFS and OS had not yet reached. At 5 years disease free survival and overall survival were 72.5% and 75% respectively. The Kaplan-Meier curves for disease free survival and overall survival has been shown in Figure 5.

Univariate analysis (Table 10):

Age and outcomes:

There were 94 patients aged <50 and 41 >50 years (median age = 47). The univariate analysis (continuous) showed a statistically significant improved outcome with increasing age for both DFS (HR: 0.96; 95% confidence interval [CI], 0.93 to 0.99; *P* = 0.036) and OS (HR: 0.95; 95% CI, 0.92 to 0.99; *P* = 0.009). By univariate (Categorical) analysis (Table 10) of age subgroups classified as age <50years and patients aged >50years, older age patients fared better than younger age patients for overall survival (Log-rank *P* = 0.011). Older age group showed a trend towards better DFS by categorical analysis but was not statistically significant (Log-rank *P* = 0.091).

Tumor grade and outcomes:

The grade was divided into two subgroups; high grade (grade 3) and low grade (grade 1 & 2). Age distribution and hormonal status in comparison with tumor grade has been shown in Table 4 & 5. Univariate analysis (Table 10) showed that tumor grade was not predictive of disease free survival (HR: 1.06; 95% CI, 0.54 to 2.06; *P* = 0.86) or overall survival (HR: 1.33; 95% CI, 0.67 to 2.67; *P* = 0.41).

Hormonal status and outcomes:

The comparison of ER status with age and pathological response has been shown in Table 6 & Table 7. Nineteen patients (25.3%) with ER positive group relapsed and died because of disease and 17 patients (29.3%) relapsed (HR: 1.24; 95% CI, 0.64 to 2.39; *P* = 0.51) and 14 (24.5%) died (HR: 1.03; 95% CI, 0.51 to 2.02; *P* = 0.97) in the ER negative group.

Similarly PR status was available for 130 out of 135 patients. Of these 65 patients were PR positive and 65 were PR negative. In PR positive group 15 patients (23%) relapsed and in PR negative group 20 (30%) relapsed (HR: 1.55; 95% CI, 0.80 to 3.01; *P* = 0.19). A total of 14 (21.8%) and 19 (29.6%) patients died in each group respectively (HR: 1.49; 95% CI, 0.75 to 2.98; *P* = 0.25).

Clinical stage and outcomes:

Stage IIIA and IIIB were the only evaluable patients that were included in the study as described in the section of patient characteristics. Overall reduction in size of the tumor and nodes was observed following chemotherapy in 129 out of 135 patients. Reduction in the size of the tumor was possible in 8 out of 9 (88.8%) patients with

cT2 tumors; 87 out of 89 (98%) patients with cT3 tumors; and 34 out of 36 (94%) patients with cT4 disease. In cT2 tumors 3 out of 9 patients relapsed and died, in cT3 tumors 17 out of 89 patients relapsed and died, and in cT4 tumor 14 out of 37 patients relapsed and died. There were no statistically significant outcome (DFS and OS) differences in any of these groups (Table 10).

Overall, 99 out of 134 patients had complete pathological absence of tumor in axillary lymph nodes. Seventy eight out of 96 patients with cN1 were ypN0 and 21 out of 38 cN2 diseased patients had pathologically sterile nodes post neoadjuvant chemoradiation. In cN1 20 out of 96 patients (20%) relapsed and with cN2 disease 16 (41%) relapsed of 39 patients (HR: 2.43; 95% CI, 1.26 to 4.69; P = 0.008). Nineteen of the cN1 relapsed patients died and 15 of cN2 relapsed patients died during the study period (HR: 2.3; 95% CI, 1.18 to 4.59; P = 0.014).

Chemotherapy and outcomes:

A total of 110 patients received FAC or FEC based chemotherapy, out of whom 39 of these achieved pCR and the remaining 71 had an incomplete response. Seven patients who had achieved pCR relapsed and 22 patients relapsed in the group who had not achieved pCR. Twenty one patients received CMF chemotherapy among which 7 achieved pCR, 4 died among those who didn't achieve pCR but none in those who achieved pCR. In our study cohort 3 patients had received Paclitaxel/epirubicin chemotherapy. All three of them are alive with one patient not achieving pCR after neoadjuvant chemoradiation. On comparison between anthracycline and CMF based chemotherapy there was no statistically significant difference in outcomes in both groups.

Pathological response and outcomes:

In 135 patients one patient progressed on neoadjuvant chemoradiation hence she was not operated upon. In the remaining 134 patients, 54 achieved tumor sterilization in breast and 80 didn't (Table 8). In the pathological complete tumor response group 13 relapsed and in incomplete response group 22 relapsed (Log-rank P = 0.713) and similarly 12 and 21 died in each group respectively (Log-rank P = 0.899).

Among the 134 patients who underwent mastectomy 28 patients had pN1, 9 patients had pN2, 2 patients had pN3 disease, and 95 patients were node negative (Table 9). Among the pN1 group 11 patients (29.3%) relapsed, among the pN2 group 4 (44.4%) relapsed, among the pN3 both patients relapsed and among pN0 18 patients (18.9%) relapsed during the follow up period. On comparison between pathological node negative and positive group there was significant survival benefit in both DFS (HR: 2.79; 95% CI, 1.44 to 5.43; *P* = 0.002) and OS (HR: 3.48; 95% CI, 1.75 to 6.91; *P* = <0.001).

A total of 47 patients achieved complete pathological response among 134 patients (One patient progressed on chemoradiation hence didn't undergo mastectomy). Among the 47 patients who achieved pCR 8 patients (17%) relapsed and among the 87 patients who didn't achieve pCR 27 relapsed (31%) (HR: 1.86; 95% CI, 0.86 to 4.16; *P* = 0.113). Only 7 out of 47 patients who attained pCR died and 26 in 87 patients who didn't achieve pCR (HR: 2.06; 95% CI, 0.89 to 4.76; *P* = 0.08).

Multivariate analysis (Table 11 & 12):

In Multivariate analysis the variables which had significant influence on survival outcomes in univariate analysis were evaluated. Among the various variables which were significant in univariate analysis, the clinical and pathological nodal stages were dependent variables hence two different models were used excluding each other. The age, clinical tumor size and pathological complete response had bearing on the outcomes and hence were taken as independent variables for analysis by Cox regression multivariate analysis of outcomes. The pathological nodal stage emerged as the strongest variable for predicting overall survival (HR: 3.15; 95% CI, 1.35 to 7.45; P = 0.009). The clinical nodal stage (HR: 2.33; 95% CI, 1.16 to 4.65; P = 0.017) and age (HR: 0.28; 95% CI, 0.10 to 0.82; P = 0.02) were also found to have significant prediction for overall survival. Surprisingly the clinical nodal stage emerged as a better variable than pathological nodal stage for correlation with disease free survival (HR: 2.44; 95% CI, 1.24 to 4.79; P = 0.009). The pathological nodal stage also a significant predictor of disease free survival (HR: 2.67; 95% CI, 1.20 to 5.89; P = 0.015). Age was not statistically significant for disease free survival unlike overall survival.

Table 2: Baseline Characteristics

Variable	N (%)
Age	
21 – 30 years	5 (3)
31 – 40 years	30 (22.2)
41 – 50 years	59 (43.7)
51 – 60 years	27 (20)
61 – 70 years	14 (10.3)
AJCC Stage	
IIIA	98 (72)
IIIB	37 (28)
IIIC	0
T stage	
T2	9 (6.6%)
T3	89 (66%)
T4	37 (27%)
N stage	
N1	96 (71%)
N2	39 (29%)
Grade	
1	2 (1.5%)
2	60 (44.5%)
3	66 (49%)
Not evaluable	7 (5%)
Histological subtypes	
Infiltrating ductal carcinoma	126 (93.3)
Medullary carcinoma	3 (2.2)
Mucinous carcinoma	3 (2.2)
Others	3 (2.2)
Hormonal status	
ER positive	75 (55.5%)
ER negative	57 (42.2%)
Hormonal status not available	3 (2.2%)

Table 3: Chemoradiation and other outcome characteristics

Variable	N (%)
Type of chemotherapy	
FAC	89 (65.9)
FEC-60	22 (16.2)
CMF	21 (15.5)
Paclitaxel + Epirubicin	3 (2.2)
Number of cycles of neoadjuvant chemotherapy	
3 cycles	8 (5.9)
4 cycles	18 (13.3)
5 cycles	5 (3)
6 cycles	104 (77)
Number of cycles of neoadjuvant chemotherapy given before radiation was started	
After 1st cycle	119 (88.1)
After 2nd cycle	9 (6.6)
After 3rd cycle	5 (3)
After 4th cycle	1 (0.7)
After 5th cycle	1 (0.7)
Pathological tumor stage	
0	54 (40)
1	46 (34)
2	30 (22.2)
3	4 (29.6)
Pathological nodal stage	
0	94 (69.6)
1	28 (20.7)
2	9 (6.6)
3	2 (1.4)
Pathological response	
pCR	47 (34.4)
No pCR	87 (64.4)

Table 4: Comparison between age and tumor grade

		Tumor grade		Total
		Low	High	
Age	<50 years	44	46	90
	>50 years	18	20	38
Total		62	66	128

Table 5: Comparison between ER and tumor grade

		Grade		Total
		Low Grade	High Grade	
ER	Positive	42	30	72
	Negative	20	35	55
Total		62	65	127

Table 6: Comparison between age and ER status

		ER		Total
		Positive	Negative	
Age	<50 years	49	44	93
	>50 years	26	14	40
Total		75	58	133

Table 7: Comparison between ER and pCR

		pCR		Total
		pCR	No pCR	
ER	Positive	19	56	75
	Negative	27	30	57
Total		46	86	132

Table 8: Comparison between clinical and pathological tumor stage

		Pathological tumor stage				Total
		0	1	2	3	
Clinical tumor stage	2	5	3	1	0	9
	3	34	30	23	2	89
	4	15	13	6	2	36
Total		54	46	30	4	134

Table 9: Comparison between clinical nodal stage and pathological nodal stage

		Pathological Node		Total
		Positive	Negative	
Clinical Node Stage	1	18	78	96
	2	21	17	38
Total		95	39	134

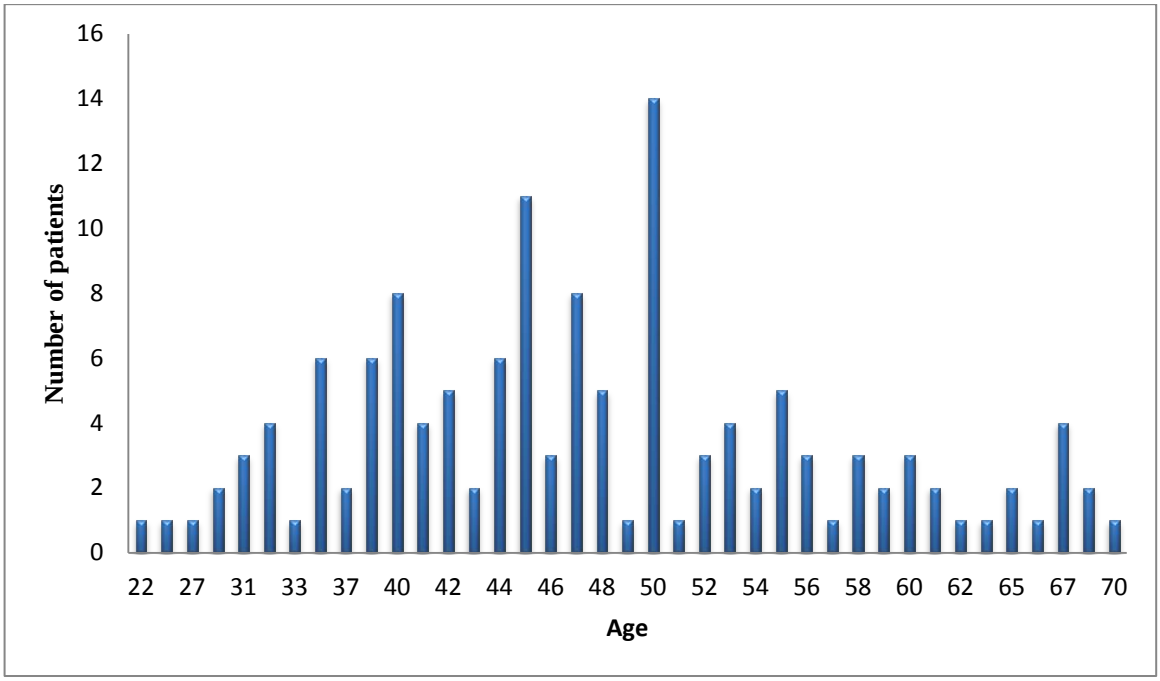

Figure 1: Age distribution

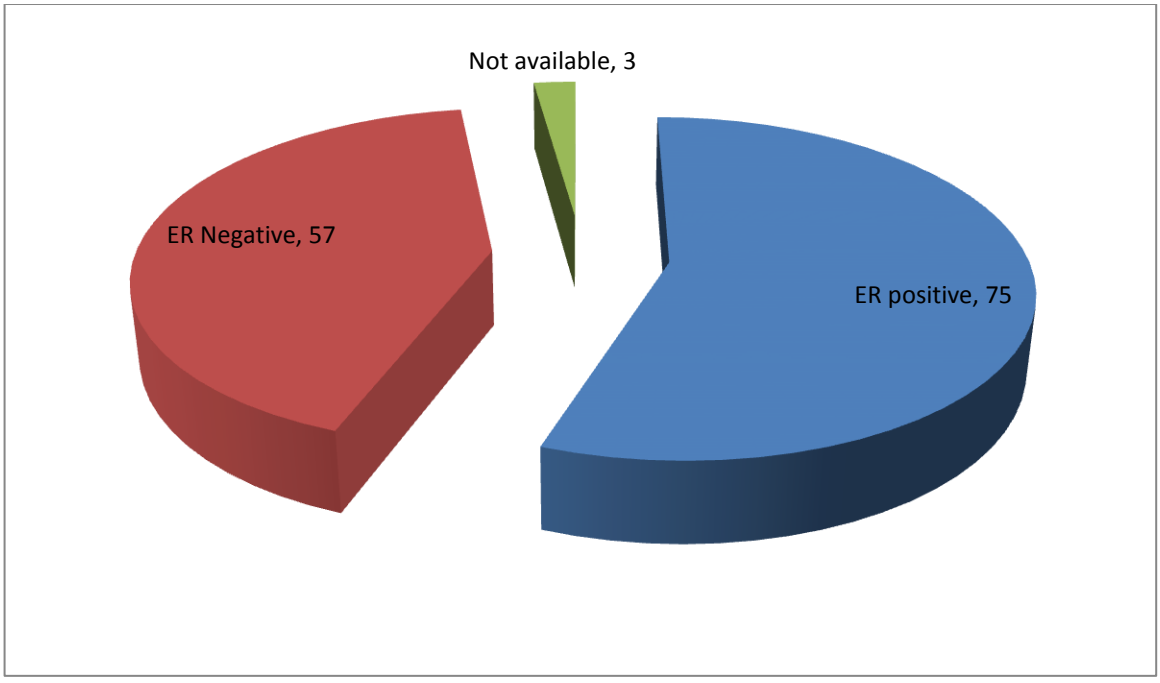

Figure 2: Hormone status

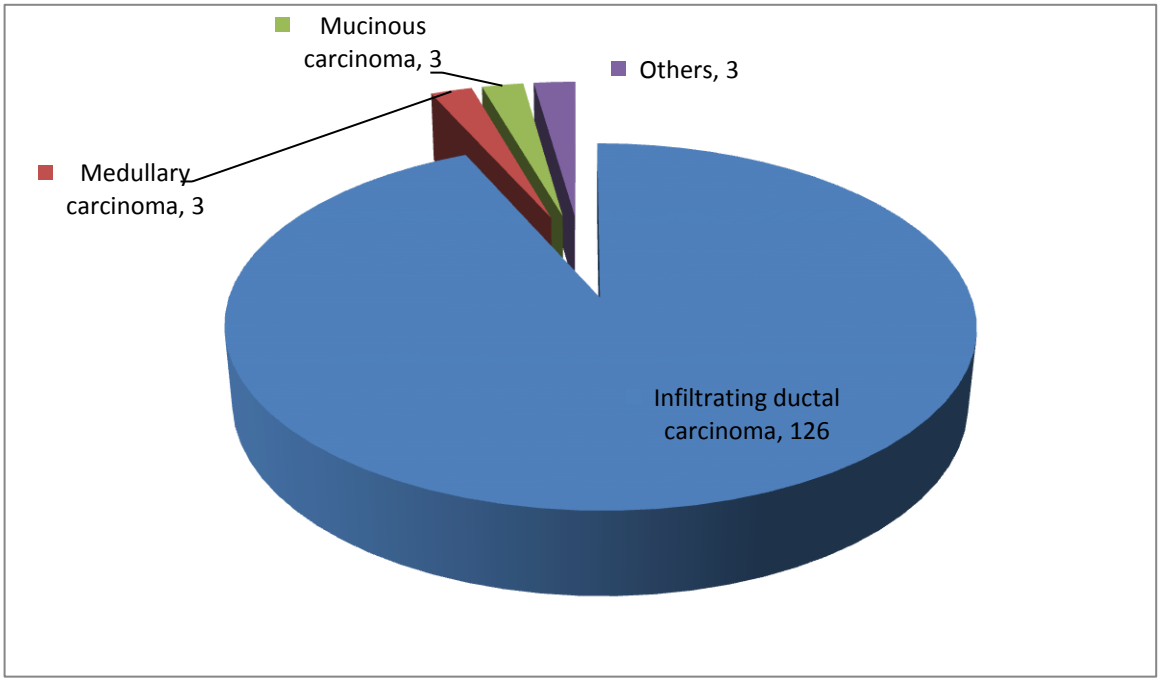

Figure 3: Histological subtypes

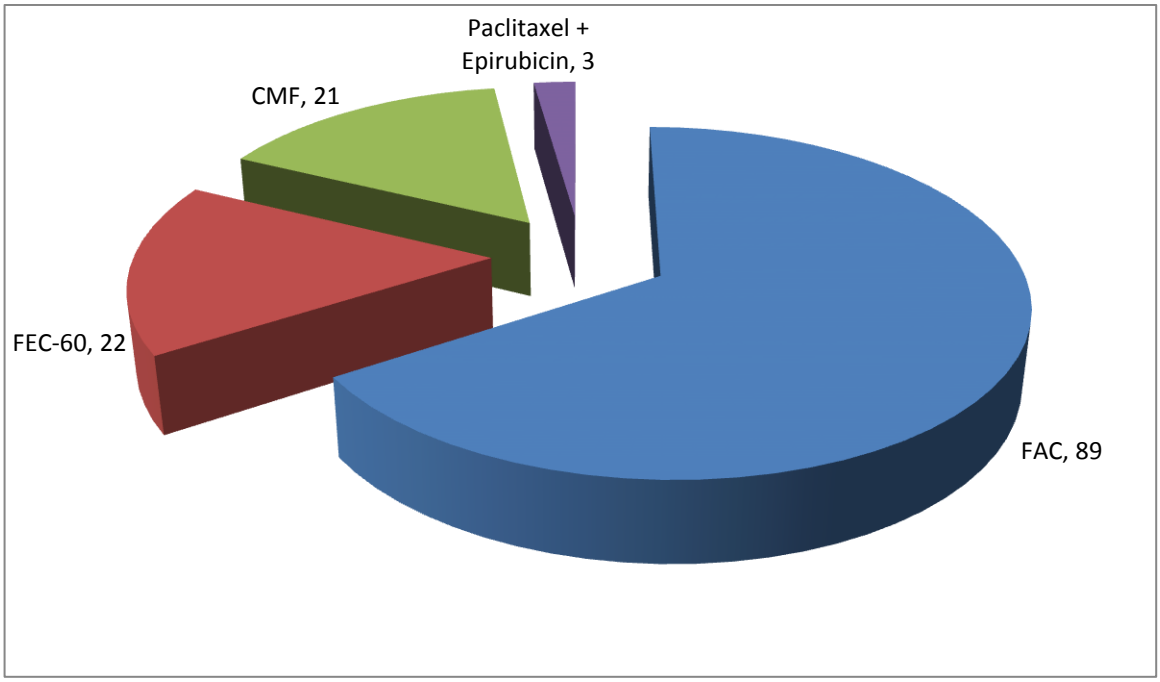

Figure 4: Type of chemotherapy

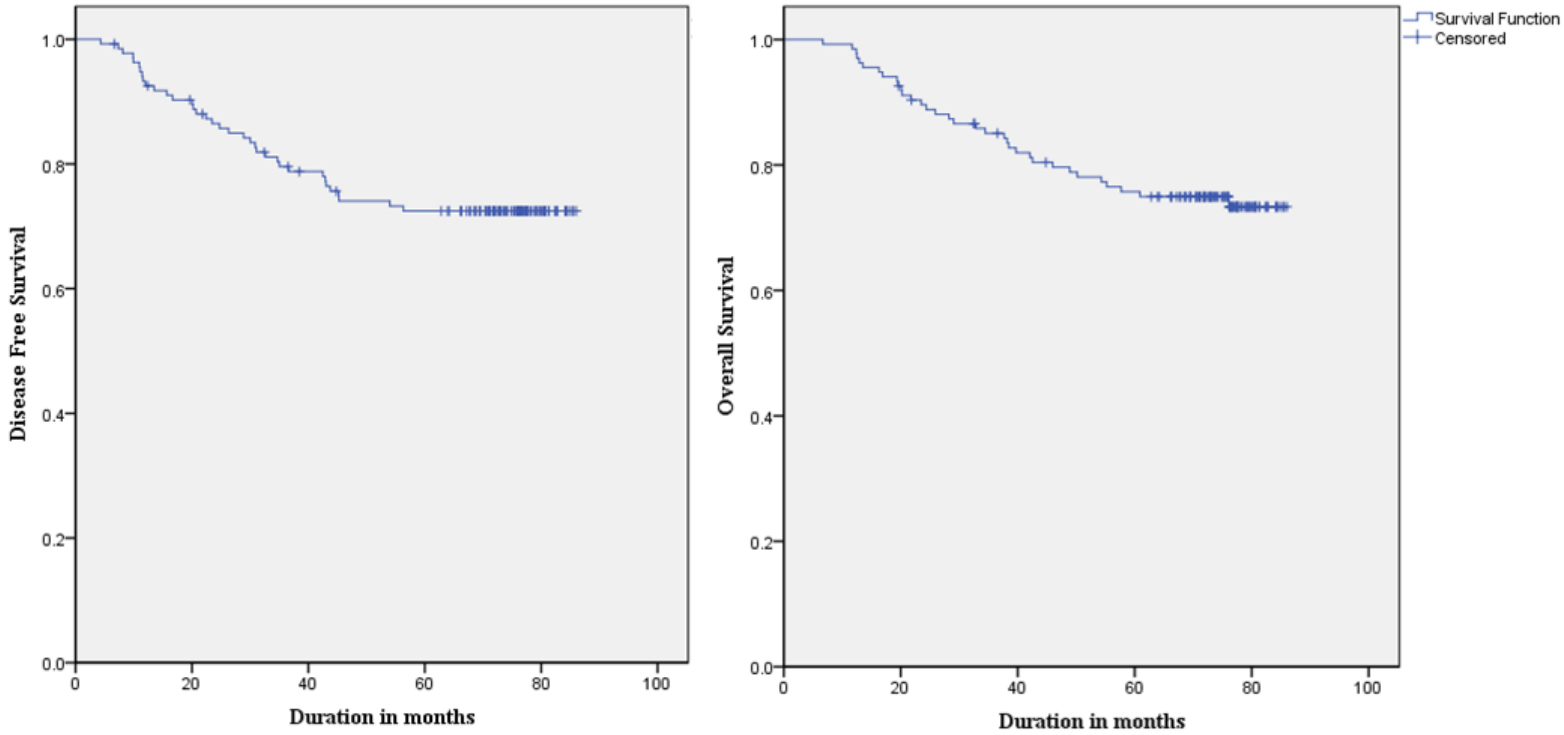

Figure 5: Disease free survival and Overall survival

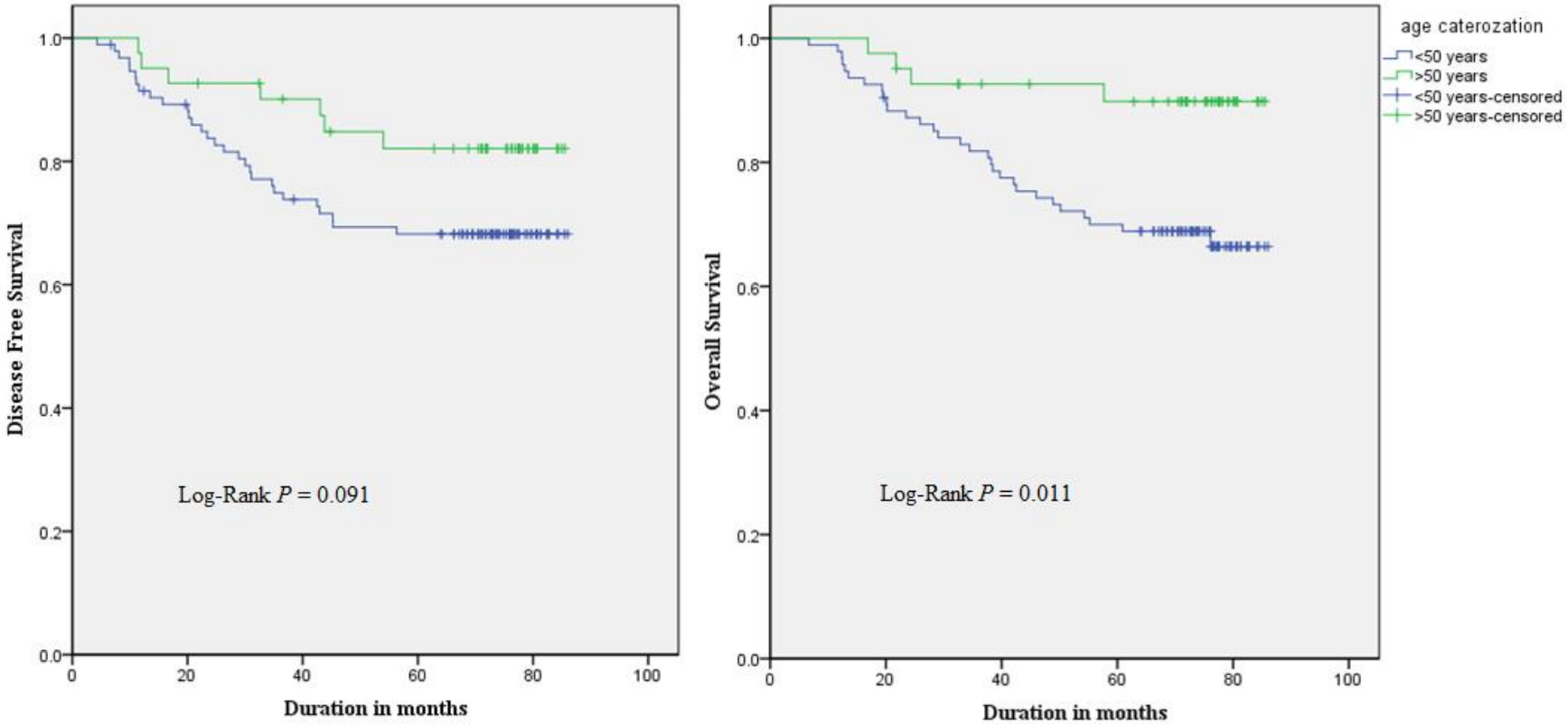

Figure 6: DFS and OS with Age

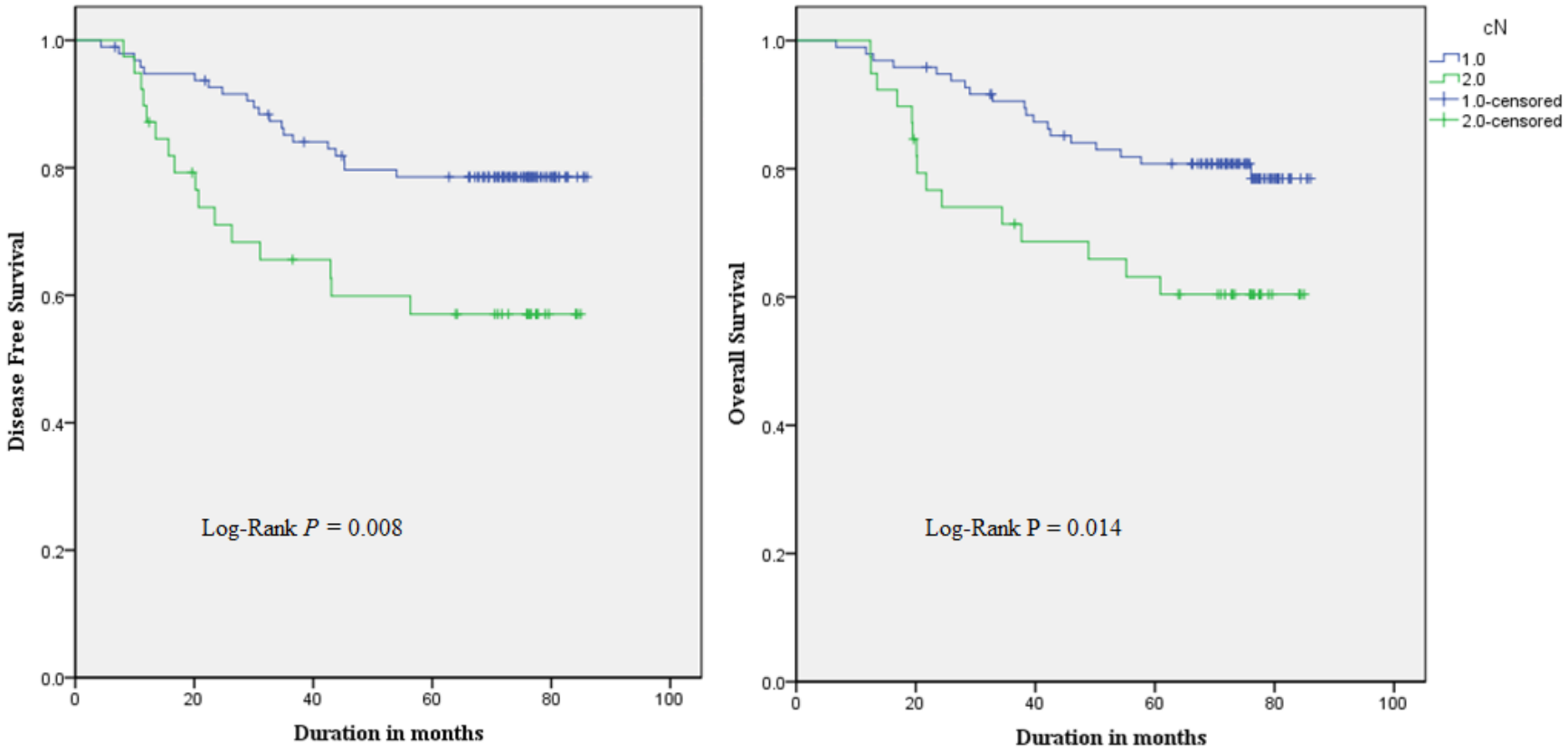

Figure 7: DFS and OS with Clinical Nodal Stage

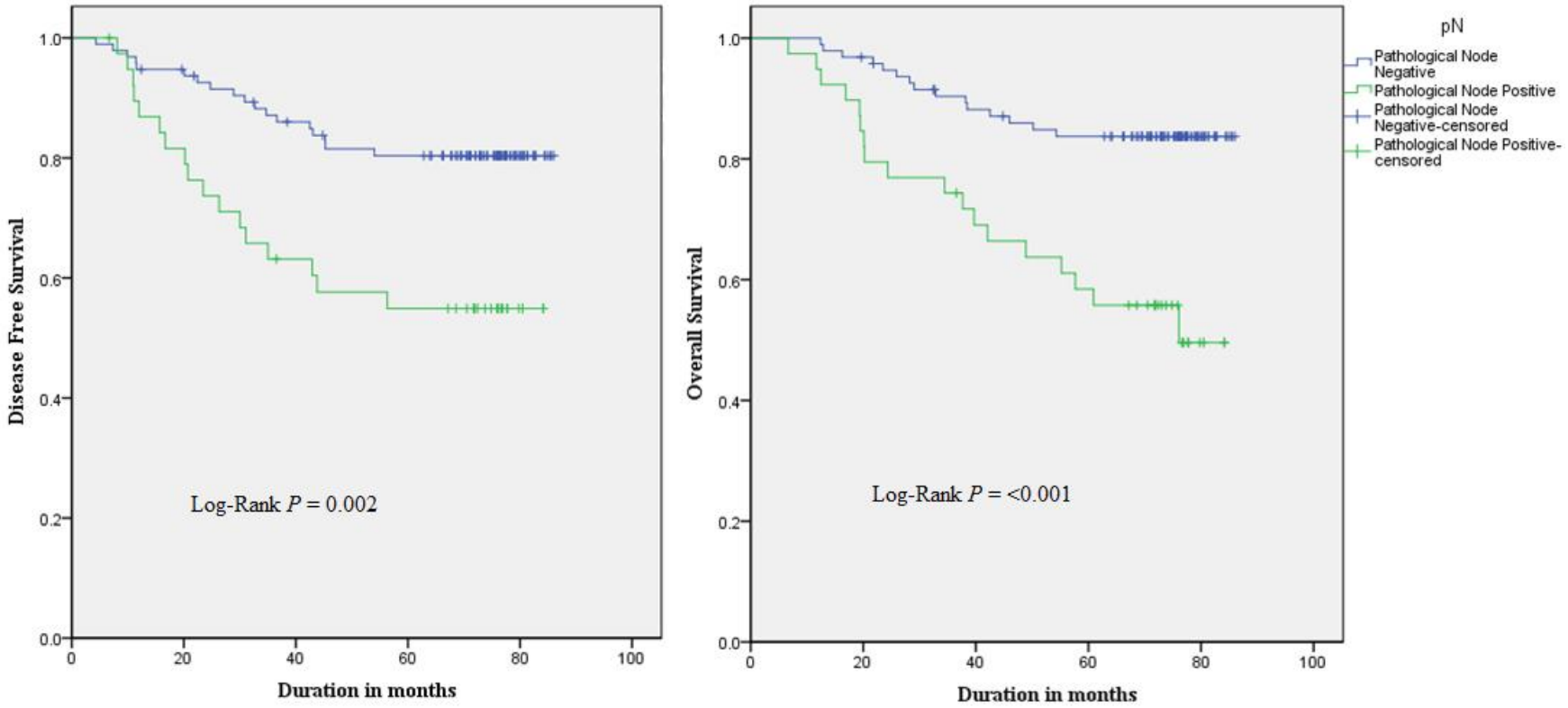

Figure 8: DFS and OS with Pathological Nodal Status

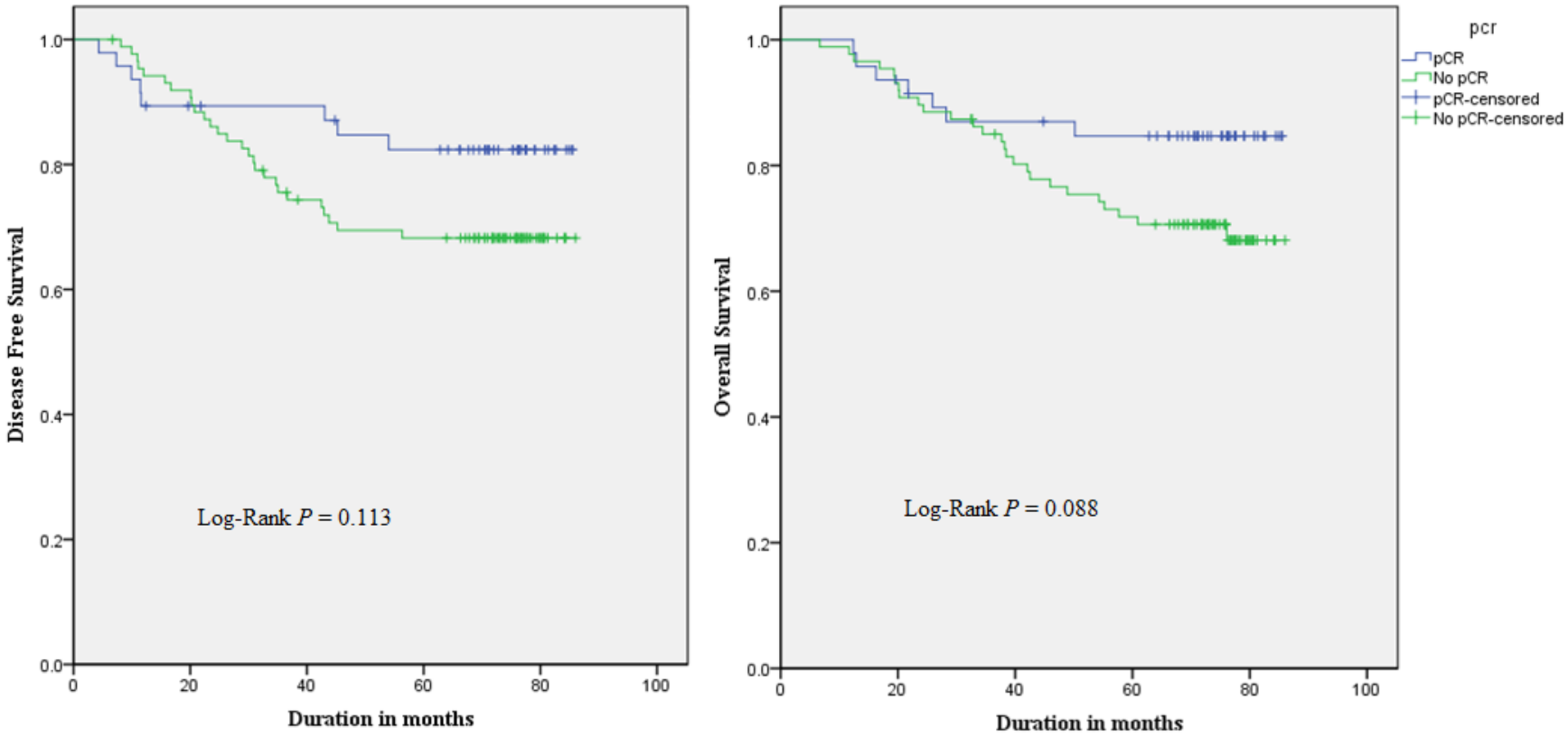

Figure 9: DFS and OS with pCR

Table 10: Univariate analysis by Cox proportional hazard models

Variable		Number	Disease Free Survival			Overall survival		
			Hazard Ratio	95% CI	P Value	Hazard Ratio	95% CI	P Value
Age (Continuous)		135	0.965	0.934 - 0.998	**0.036**	0.955	0.922 - 0.989	**0.009**
Age (Categorical)	**<50 years**	94	1	0.218 – 1.136	0.097	1	0.099 – 0.802	**0.018**
	>50 years	41	0.497			0.282		
Clinical Tumor stage		135	1.120	0.603 – 2.082	0.720	1.668	0.888 – 3.135	0.112
Clinical Node Stage	**1**	96	1	1.255 – 4.689	**0.008**	1	1.184 – 4.592	**0.014**
	2	39	2.426			2.331		
Clinical stage	**IIIA**	98	1	0.645 – 2.670	0.453	1	1.102 – 4.327	**0.025**
	IIIB	37	1.313			2.184		
Grade	**Low**	62	1	0.546 – 2.057	0.864	1	0.668 – 2.660	0.414
	High	66	1.060			1.333		
ER	**Positive**	75	1	0.646 – 2.393	0.514	1	0.508 – 2.02	0.972
	Negative	58	1.244			1.013		
PR	**Positive**	65	1	0.801 – 3.015	0.193	1	0.749 – 2.980	0.255
	Negative	65	1.554			1.493		
Neoadjuvant CT+RT	**Anthra based**	114	1	0.239 – 1.908	0.458	1	0.266 – 2.141	0.596
	CMF	21	0.675			0.596		
Number of cycles of neoadjuvant chemotherapy		135	1.565	0.943 – 2.596	0.083	1.429	0.887 – 2.301	0.142
pN	**Negative**	95	1	1.441 – 5.436	**0.002**	1	1.753 – 6.916	**<0.001**
	Positive	39	2.798			3.482		
pCR	**Yes**	47	1	0.859 – 4.167	0.113	1	0.897 – 4.764	0.088
	No	87	1.892			2.067		

Table 11: Multivariate analysis for DFS by Cox proportional hazard models

Variable	Model 1			Model 2		
	Hazard Ratio	**95% CI**	**P Value**	**Hazard Ratio**	**95% CI**	**P Value**
Age	0.508	0.222 – 1.163	0.109	0.518	0.226 – 1.186	0.120
Clinical Tumor stage	1.183	0.665 – 2.106	0.568	0.961	0.529 – 1.746	0.895
Clinical Node Stage	2.445	1.246 – 4.798	0.009			
pN				2.667	1.207 – 5.889	0.015
pCR	1.974	0.893 – 4.363	0.093	1.119	0.439 – 2.850	0.814

Table 12: Multivariate analysis for OS by Cox proportional hazard models

Variable	Model 1			Model 2		
	Hazard Ratio	**95% CI**	**P Value**	**Hazard Ratio**	**95% CI**	**P Value**
Age	0.287	0.101 – 0.819	0.020	0.293	0.103 – 0.836	0.022
Clinical stage	1.687	0.920 – 3.090	0.091	1.326	0.710 – 2.476	0.377
Clinical Node Stage	2.328	1.163 – 4.656	0.017			
pN				3.155	1.335 – 7.452	0.009
pCR	2.145	0.928 – 4.960	0.074	1.062	0.382 – 2.953	0.908

Chapter 4

Evaluation of tumor angiogenesis by CD31

Chapter 4: Evaluation of tumor angiogenesis by CD31

Paraffin blocks of 55 patients' core needle biopsy could be retrieved form the tumor bank out of the 135 patients in the study. Positive staining for CD31 was observed in 43 samples. The tumor tissue was inadequate for immunohistochemical staining for five samples. Seven samples had no expression of CD31 on immunohistochemical staining. These 7 samples were restained for CD31 and were still negative. They were thus excluded from study.

The Chalkley method of counting the blood vessels could not be applied because the biopsy tissue had inadequate number of hotspots. Thus all the blood vessels in tissue sample were counted. The number of blood vessels was counted by two independent observers. The average count of the two observers was considered for statistical analysis. The median number of blood vessels with CD31 expression was 52 and the mean value was 91.

The median value of 51 was considered for statistical analysis. The blood vessel count ranges from 3 to 391. Thus mean value can distort the statistical analysis. Hence the median value of 51 was considered for analysis

The number of patients with blood vessel count lower than the median was 22. Sixty eight percent of these patients were ER positive and 52% were PR positive. Fifteen tumors among them were high grade (grade 3) and 7 were low grade (grade 2). There were 14 Stage IIIA and 8 stage IIIB patients. Similarly, the number of patients with blood vessel count higher than median was 21. Among them 62% and 50% were ER and PR positive respectively. This category contained 9 high grade

and 12 low grade tumors. Fourteen of them were stage IIIA and 7 were stage IIIB patients.

The pathological complete responses were observed in 9 patients in both the groups of low and high blood vessel count and 12 didn't have complete pathological response. One patient progressed on treatment with neoadjuvant chemoradiation and hence didn't undergo surgery. This patient was having a CD31 blood vessel count of 30. The univariate analysis (Table 13) for survival outcomes was performed by classifying the patients into two groups (≤52 and >52). The high CD31 count didn't had a statistically significant correlation with either DFS (HR: 0.558; 95% CI, 0.19 to 2.42; $P = 0.56$) or OS (HR: 0.386; 95% CI, 0.10 to 1.46; $P = 0.16$).

Table 13: CD31 Count Data

SL No	Index No	Stage	Pathology	Grade	ER	PR	CD31 Count
1	217	IIIB	1	3	1	2	37
2	226	IIIB	4	3	1	1	52
3	227	IIIA	1	2	2	2	290
4	228	IIIA	1	2	1	1	286
5	233	IIIA	1	2	1	1	286
6	235	IIIB	1	3	1	1	107
7	238	IIIA	1	2	1	1	146
8	240	IIIA	1	3	2	NA	37
9	293	IIIA	1	3	1	1	5
10	296	IIIA	1	3	1	2	347
11	355	IIIA	1	2	2	2	13
12	369	IIIA	1	3	2	2	175
13	415	IIIB	1	3	2	2	136
14	455	IIIB	1	3	2	2	3
15	480	IIIA	1	3	2	1	6
16	493	IIIA	4	3	1	1	8
17	500	IIIB	1	3	2	2	84
18	515	IIIA	1	2	1	1	40
19	528	IIIB	1	3	2	2	30
20	531	IIIA	1	2	1	2	9
21	539	IIIA	1	2	1	1	75
22	545	IIIB	1	2	1	1	48
23	550	IIIA	1	3	1	1	81
24	552	IIIA	1	2	1	1	9
25	558	IIIB	1	2	1	1	233
26	568	IIIB	1	2	1	NA	55
27	574	IIIB	1	3	1	1	9
28	575	IIIA	1	2	1	2	3
29	576	IIIA	1	3	2	2	24
30	583	IIIA	1	3	1	1	46
31	591	IIIA	1	3	1	1	114
32	615	IIIB	1	2	1	1	7
33	616	IIIB	1	2	1	2	245
34	622	IIIA	1	3	1	1	11
35	629	IIIA	3	NA	1	2	97
36	639	IIIB	4	3	1	1	18
37	673	IIIA	1	3	2	2	13
38	684	IIIA	1	3	2	2	85
39	686	IIIA	1	2	2	2	391
40	702	IIIA	1	2	2	2	221
41	714	IIIA	1	3	1	1	73
42	715	IIIA	1	3	1	2	26
43	737	IIIB	1	2	2	1	156
Stage: AJCC Clinical stage; Pathology: 1- Infiltrating ductal carcinoma; 2- Medullary carcinoma; 3-Mucinous carcinoma; 4 - Others); NA: Not available							

Table 14: Univariate analysis of CD31

Variable		Number	Disease Free Survival			Overall survival		
			Hazard Ratio	95% CI	P Value	Hazard Ratio	95% CI	P Value
CD31 Blood	**≤52**	22	1	0.193 – 2.429	0.558	1	0.102 – 1.457	0.160
Vessel Count	**>52**	21	0.685			0.386		

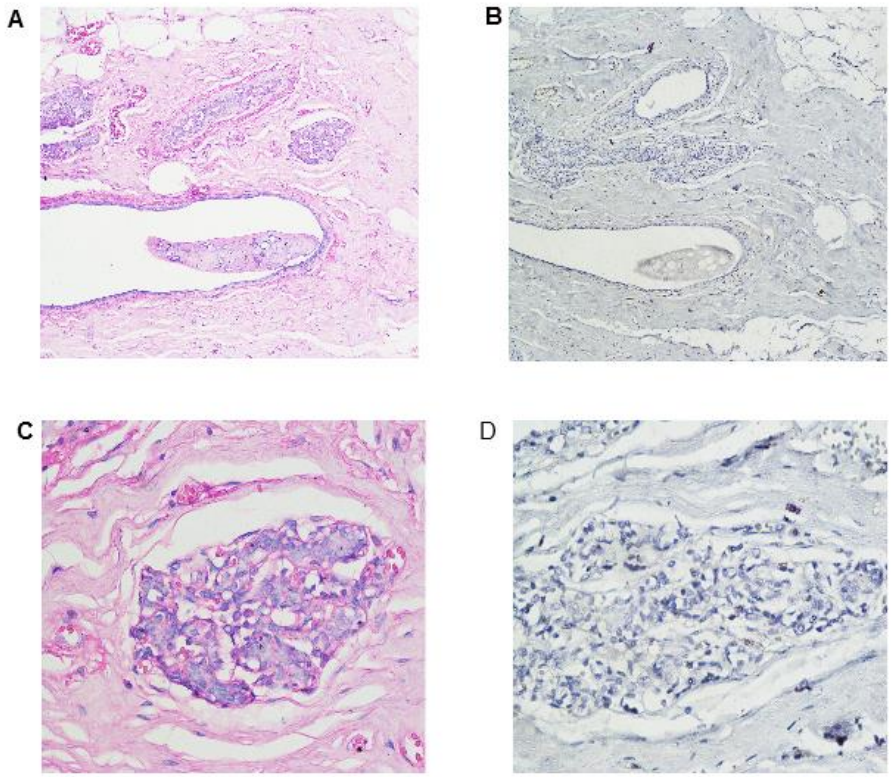

Figure 10: Expression of CD31 in Normal Breast Tissue Hematoxylin and Eosin stain and CD31 under magnification 10x (A & B), and 40x (C & D) respectively. The CD31 count was faintly expressed.

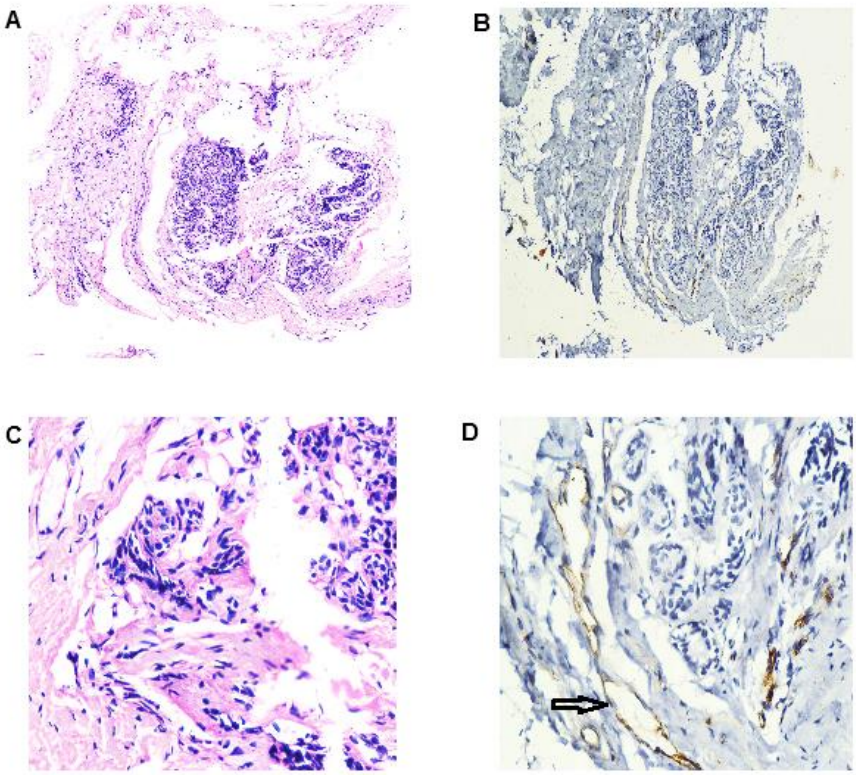

Figure 11: Low expression of CD31 in LABC tumor tissue Hematoxylin and Eosin stain and CD31 under magnification 10x (A & B), 40x (C & D). The CD31 count expression is low in this tumor tissue (arrow).

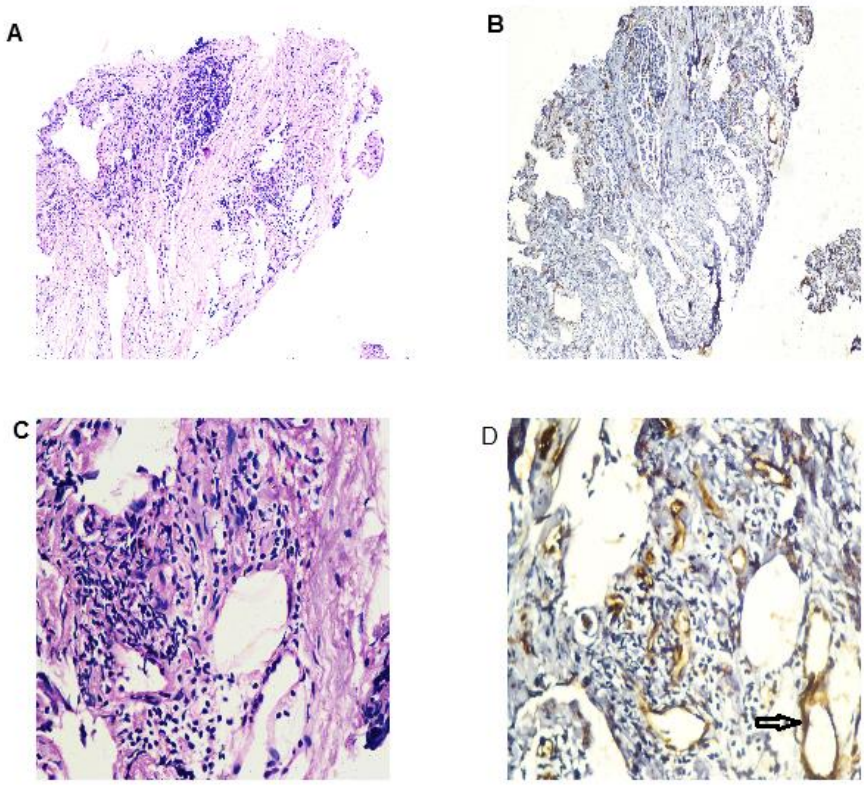

Figure 12: High expression of CD31 in LABC tumor tissue Hematoxylin and Eosin stain and CD31 under magnification 10x (A & B), 40x (C & D). The CD31 expression was high in this tumor tissue (arrow).

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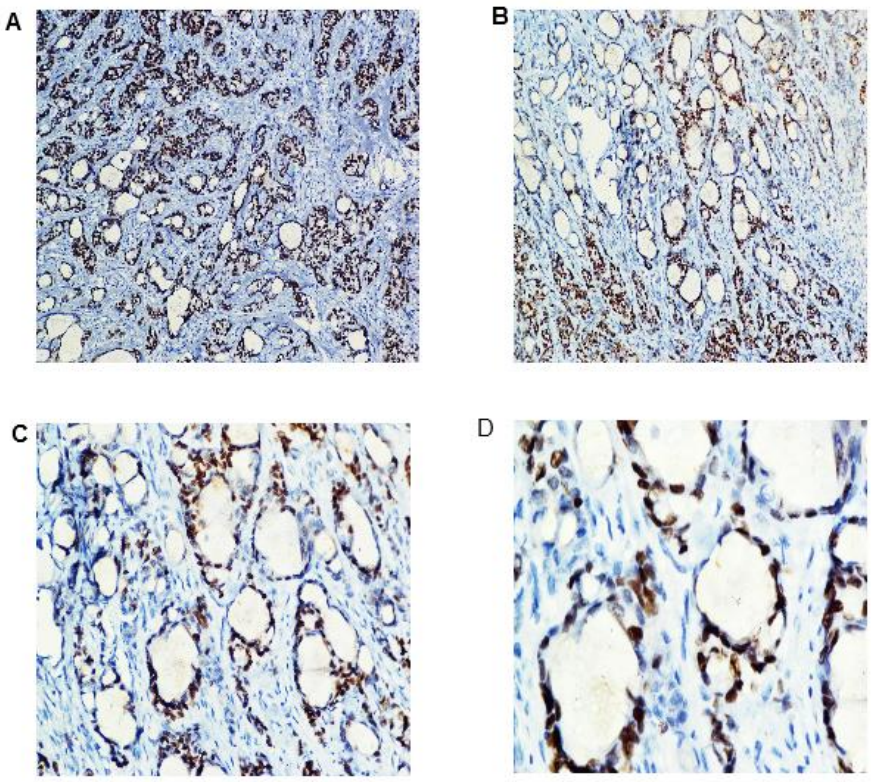

Figure 13: Expression of ER and PR in LABC tumor tissue Hormonal receptor expression of ER and PR under various magnifications ER (A: 10x; C: 40x) and PR (B: 10x; D: 40x)

Chapter 5: Discussion

Chapter 5: Discussion

The prevalence of LABC is high (152) among women in Indian subcontinent compared to developed countries (153) because of poor socioeconomic factors, education and awareness towards general health and in particular for breast cancer. Treatment of LABC by neoadjuvant chemotherapy to shrink the tumor and bring it within purview of mastectomy is routinely performed globally. However, treatment by neoadjuvant concurrent chemoradiation has not been routine and is followed in only few centres globally. The various studies across the world have shown an overall survival between 50 – 60% at 5 years in patients with locally advanced breast cancer (153). In our hospital by using concurrent chemoradiation in neoadjuvant setting we could achieve an overall survival of about 75% at 5 years.

In our study, young patients had a worse prognosis compared to older which was statistically significant. This poor prognosis could not be explained by the grade or hormonal status as there were near equal number of patients in each of these groups. Our study did not show any difference in survival between patients with higher grade or hormonal positivity. This is in contrast to major trials on breast cancers that have shown better survival in ER positive subgroup of patients by use of adjuvant hormonal therapy (154–156). We did not observe any statistically significant difference in outcomes between anthracycline based or CMF based chemotherapy when added concurrent to radiotherapy. Probably the concurrent radiation nullifies the advantage of anthracycline over CMF based chemotherapy but the number of cases are too less to substantiate this and needs to be prospectively studied in a randomized trial.

Though higher pCR was seen in the ER negative subgroup of patients (ER negative: 27; ER positive: 19) this did not translate to improved outcomes. It has been already shown that ER negative breast tumors have increased sensitivity to chemotherapy and has been attributed in part to higher grade of these tumors (157,158) but in our study there were near equal number of patients in both subgroups (ER positive: 30; ER negative: 35). Hence we could not explain the reason for increased pCR in this subset. If c-erb2 status would have been available the role of triple negative breast tumors in achieving pCR would have clarified. In NSABP B-27 trial the pCR rate increased to 26% by addition of docetaxel to AC regimen (22). The complete pathological response rate in our study was 35% which was superior to the NSABP B-27 trial. On univariate analysis, the role of clinical and pathological tumor stage did have little influence in the survival outcomes. But achievement of pathological complete response in lymph nodes strongly influenced the survival outcomes. Interestingly clinical nodal size also had a statistically significant prognostic value in predicting the disease free and overall survival which was shown by Cox regression analysis.

There were potential limitations of this study. These were that it was a retrospective analysis of clinical data; menopausal status and expression of c-erbB2 in the tumors were not available. Overall, with a median follow up of 6 years, the numbers of events were less and this had an impact on analysis of prognostic factors and relating it to outcomes.

Our study is unique in considering the use of concurrent radiation along with neoadjuvant chemotherapy and we could demonstrate an improved disease free and

overall survival. This approach is tailored to the average patient presenting at public hospitals. The impressive survival of 75% at five years compares favourably with any other approach. If one evaluates Adjuvant online with the one set of clinical factors as found in this study the overall survival is 72% at 10 years (median age 47, T3, Grade II, ER positive and N1). Alternatively, with another set of clinical factors (median age 47, T3, Grade III, ER negative and N2) then the 10 year survival is 32%. As within this study both groups are present, the overall average as predicted by Adjuvant online would be approximately 52%. Thus our outcome in this clinically challenging group of patients is not inferior and it remains to be seen whether it will be superior. However, this approach is not devoid of side effects. Addition of radiation during chemotherapy has been associated with increased morbidity in terms of local site skin reaction and prolonged chemotherapy induced myleosuppression. However morbidity associated with chemoradiation has to be documented prospectively and in a randomized trial comparing it with chemotherapy alone.

Angiogenesis

CD31 immunohistochemistry was performed on tumors from 43 patients in the 55 trucut biopsy slides procured from the tumor bank. The tumor tissue was inadequate for immunohistochemical staining for CD31 in 5 patients and was negative in 7 (16%) biopsy tissues (repeated twice). The published literature documents that negative results can be as high as 20% (159). Considering, that our tissues were over 6 years old the 16% negative rate can partially be explained by their deterioration of their quality over the years during storage.

The studies investigating angiogenesis in breast cancer have been done using various markers like CD31, FVIII, CD34 and CD105. Most of these studies have been done on the mastectomy specimen where there is adequate tissue for IHC staining. They have assessed the microvascular density with outcomes when patients were treated with adjuvant chemotherapy. Guidi et al (160) evaluated the role of MVD by employing expression of FVIII as a marker in node positive breast cancer from the breast tissues of participants form the CALBG 8541 study. These patients had received adjuvant CAF chemotherapy. The study could not find any significant association of MVD with relapse-free or OS outcomes. This study however found that prominent plexiform vascular patter was associated with decreased OS (P = 0.0085) by univariate analysis. Similar results with FVIII antigen and outcomes were found with another large study involving 685 patients (161). The largest study on CD34 antigen used Chalkley method for counting of blood vessels (162). The study showed a positive correlation between high expression of CD34 with higher tumor size, grade, and nodal metastases. Gasparini et al (147) studied the role of CD31 in 271 node-positive and 260 node-negative patients. The statistically significant correlation was found between CD31 and outcomes which was not with p53 or hormonal receptors used in the study. CD105 being more specific marker for endothelial tissue has been found to have better correlation with DFS and OS than CD34 (163).

A meta-analysis on the significance of microvessel density in breast cancer was published in 2004 (159). The meta-analysis showed that microvascular density had an inverse relationship with survival outcomes with vascularity assessed by

CD31 and CD34 expression emerging as strong prognostic indicators. The commonly used method to count the blood vessels is the Chalkley method because of its simple methodology and reproducibility (159).

As there was relatively less tumor tissue on the core biopsy specimen in our study we had to resort to counting the entire tissue slide rather than 'hot spots'. On comparison with grade of tumors, our results showed that low CD31 rather than high CD31 count was more likely to be associated with higher grade (68.2% versus 45%) compared to other studies. We could also not demonstrate statistically significant difference in outcome between high and low MVD as determined by CD31 expressions. This partly because we have evaluated only a third of tumors and the number of events in the whole cohort is small. We are continuing the study and plan to complete immunohistochemistry on all the 135 patients' core needle biopsy slides. The generation of additional data will in all probability establish further the role of MVD in LABC. To our best knowledge, this may be the first study on the tumor blood vessels density in patients with locally advanced breast cancer in India and globally.

Future directions

Since the emergence of role of tumor angiogenesis in the pathogenesis of cancer, there has been increasing research into newer tumor blood vessel specific markers. One such specific marker is CLEC14a which belongs to thrombomodulin family of receptors (164). It has been found to be specifically expressed in tumor endothelial cells. It helps in formation of filopodia and migration of endothelial cells in neo-angiogenesis of tumor blood vessel (90). Low shear stress due to poor blood flow in

the disorganized tumor vasculature has been proposed to induce expression of CLEC14a on tumor vessels and pro-angiogenic phenotypes. CLEC14a has been found to be strongly expressed on tumor vasculature compared with vasculature in healthy tissue (90). Studies on such tumor endothelial cell specific markers may pave way for research into designing targeted therapies to tumor endothelial cells sparing the normal vasculature.

There are newer drugs being developed targeting angiogenesis in the field of oncology. Bevacizumab though was given accelerated approval initially by FDA it was withdrawn later as it could not show any benefit of survival. Several multi-kinase inhibitors have been approved in various malignancies e.g., Sunitinib, Pazopinib and Regorafenib. Aflibercept being a fusion protein containing the extracellular domains of VEGF R1 & R2 have been found to be beneficial in metastatic colorectal cancers. But little advances have been made in the field of breast cancer targeting angiogenesis. Discovery of newer specific tumor angiogenic markers may pave way for specific targeted therapies in the future.

Chapter 6: Conclusions

1. The use of concurrent radiation to chemotherapy in neoadjuvant setting is feasible. It can increase the survival rates and needs to be studied prospectively in randomized control trials.
2. Though a retrospective data, our study did show a strong association between pathological nodal stage and outcome in patients with locally advanced breast cancers. Also younger age is associated with poorer prognosis.
3. Our study established that further research is required to assess the importance of tumor angiogenesis as a predictive factor for outcomes in patients with locally advanced breast cancer.

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Appendix

Proforma for data collection

Index No:

UHID No:

OP No:

Patient name:

Age:

Date of admission:

Clinical stage

1. Tumor size:
2. Nodal size:
3. Metastasis: (Y/N):

Pathology

1. Biopsy No
2. Date of reporting of biopsy:
3. Pathology
4. Grade
5. ER
6. PR
7. C-erb B2

Neoadjuvant chemotherapy

1. Type of chemotherapy
2. Date of start of chemotherapy
3. Number of cycles of chemotherapy before surgery

Neoadjuvant radiation

1. Date of start of radiation
2. Dose

Mastectomy

1. Date of surgery:
2. Type of surgery:

Pathological stage

1. Pathological tumor size:
2. Pathological nodes positive:
3. Pathological nodes removed:
4. Pathological tumor stage:
5. Pathological nodal stage:

Adjuvant treatment

1. Adjuvant chemotherapy
    a. Date of start of chemotherapy
    b. Type of chemotherapy
    c. Number of cycles of chemotherapy
    d. Date of completion of radiation
2. Adjuvant radiation
    a. Date of start of radiation
    b. Dose
    c. Area
    d. Date of completion of radiation

Adjuvant hormonal therapy:

Relapse

1. Date of relapse
2. Site of relapse

Date of expired:

Date of last follow-up:

Clinical data

Abbreviations:

T: Clinical Tumor stage

N: Clinical Nodal stage

DOD: Date of diagnosis

BX: Histopathology of trucut biopsy

1. Infiltrating ductal carcinoma
2. Mucinous carcinoma
3. Medullary carcinoma
4. Others

Gr: Grade of tumor

ER: Estrogen receptor

PR: Progesterone receptor

NACT: Neoadjuvant chemotherapy

Cyc: Number of neoadjuvant chemotherapy cycles

RT: Radiation therapy

MRM: Date of modified radical mastectomy

pT: Pathological tumor size

pN: Pathological nodal positive number

ADJ CT: Adjuvant chemotherapy

cyc: Adjuvant chemotherapy cycles

AJ RT: Adjuvant radiation

SL NO	INDEX	AGE	T	N	DOD	BX	Gr	ER	PR	NACT	Cyc	RT	MRM	pT	pN	Node dissected	ADJ CT	cyc	AJ RT	Relapsed date	Expired date	Last follow up
1	6	47	3	2	29-12-2006	1	2	2	2	4	6	1	22-05-2007	0	0	9			IMR			09-08-2008
2	13	56	3	1	03-01-2007	1	3	2	2	1	6	1	15-06-2007	1	0	10			IMR			22-08-2013
3	21	45	3	1	10-01-2007	1	3	2	2	1	5	1	01-05-2007	0	0	13	1	1	IMR			19-10-2013
4	23	50	3	1	12-01-2007	1	2	1	1	1	6	1	29-06-2007	2	5	18			IMR			02-02-2013
5	25	29	3	1	06-01-2007	1	1	1	1	1	6	1	14-06-2007	2	0	6			IMR			26-10-2013
6	26	44	3	1	05-01-2007	1	3	2	2	1	6	1	08-06-2007	1	0	19			IMR			11-05-2013
7	36	38	3	1	13-01-2007	1	2	1	1	3	6	1	19-06-2007	1	0	13			IMR	01-10-2010		24-07-2013
8	48	52	3	1	26-01-2007	3	1	1	1	1	6	1	02-10-2007	3	0	14			IMR			31-08-2013
9	49	40	3	1	29-12-2006	1	3	2	2	1	6	1	24-07-2007	1	0	13						22-01-2014
10	51	42	2	2	26-01-2007	1	2	2	2	1	4	1	30-04-2007	1	0	17						14-05-2013
11	53	42	4	2	03-02-2007	1	2	1	1	1	6	1	26-06-2007	1	4	6			IMR			01-01-2014
12	61	44	4	2	26-01-2007	1	2	2	2	1	6	1	22-07-2008	2	3	16				23-09-2008	23-09-2008	23-09-2008
13	65	45	3	1	10-01-2007	1	3	1	1	1	6	1	29-06-2007	0	0	14						16-09-2013
14	71	41	3	1	13-02-2007	2		2	2	1	3	1	30-04-2007	0	0	20	1	3				01-10-2013
15	79	67	2	2	12-02-2007	1	3	1	1	3	6	1	08-05-2007	2	1	8	3	3	IMR	27-06-2008	11-02-2009	11-02-2009
16	81	67	2	2	21-02-2007	1	3	2	2	3	5	1	15-06-2007	0	0	16	3	1				13-02-2014
17	83	51	3	1	08-02-2007	1	3	2	2	1	6	2	06-07-2007	2	0	11			IMR			11-07-2013
18	86	56	3	1	21-02-2007	1	2	1	1	2	4	1	14-05-2007	1	2	8			IMR			16-01-2013
19	92	54	3	2	23-02-2007	1	3	2	2	4	3	2	04-06-2007	0	0	9	4	3	IMR	06-09-2010		04-03-2013
20	100	65	4	2	02-05-2007	1	3	2	2	3	6	1	17-09-2007	1	4	7				26-04-2008	19-09-2008	19-09-2008
21	111	31	3	1	14-02-2007	1		2	2	1	6	1	22-08-2007	2	0	10			IMR			20-03-2013
22	124	35	4	1	20-02-2007	1	2	2	2	1	6	1	26-07-2007	1	0	13			IMR	03-09-2009	01-11-2009	01-11-2009
23	127	55	3	1	06-03-2007	1	2	1	1	1	3	1	07-06-2007	0	0	15	1	3	IMR			03-05-2012
24	133	53	2	2	06-03-2007	1	3	1	1	1	4	1	13-07-2007	0	1	7						23-07-2013
25	143	63	3	1	06-03-2007	1	2	2	2	1	4	2	25-06-2007	1	0	13	1	2				10-06-2013
26	145	50	3	1	07-02-2007	1	3	2	2	1	6	1	03-08-2007	0	0	12			IMR			13-02-2014
27	158	66	4	2	03-03-2007	1	3	1	1	3	6	1	04-09-2007	1	3	8			IMR			30-01-2014
28	159	35	3	1	19-03-2007	1	3	2	2	1	6	1	30-08-2007	0	0	3				10-01-2008	04-05-2009	04-05-2009
29	162	59	3	1	09-03-2007	2	3	1	1	1	3	1	29-06-2007	0	0	12	1	3	IMR			24-07-2013
30	170	61	3	1	14-03-2007	1	3	2	2	2	6	1	10-09-2007	0	0	11			IMR			18-06-2013
31	171	32	3	1	26-03-2007	1	3	2	2	2	6	1	18-09-2007	0	0	3						08-01-2014
32	174	57	4	1	09-03-2007	1	3	1	1	2	6	1	31-08-2007	0	0	10						17-03-2014
33	178	55	3	2	28-03-2007	1	2	1	1	2	4	1	09-07-2007	2	3	17	2	2	IMR			10-01-2013
34	191	32	3	1	19-03-2007	1		1	1	1	6	1	25-09-2007	1	2	6			IMR	02-02-2010	01-09-2010	01-09-2010
35	193	32	3	1	05-04-2007	1	2	2	2	1	6	1	01-10-2007	2	0	20			IMR	01-10-2010	13-01-2011	13-01-2011
36	195	60	4	1	14-03-2007	1	3	2	2	1	4	1	07-08-2007	1	2	9	1	2				08-07-2013
37	209	69	3	1	12-04-2007	1	3	1		1	4	1	31-07-2007	1	0	10						17-03-2014
38	214	47	3	2	30-07-2007	2		2	2	1	6	1	22-01-2008	2	0	8			IMR			24-10-2013
39	217	45	4	2	11-08-2007	1	3	1	2	3	6	2	19-12-2007	0	0	13			IMR	DEFAULTED	17-08-2008	17-08-2008
40	226	48	4	1	08-08-2007	4	3	1	1	1	6	1	18-02-2008	2	2	11			IMR			26-02-2014
41	227	44	3	2	11-08-2007	1	2	2	2	1	6	1	28-01-2008	2	0	8			IMR			10-11-2012
42	228	48	3	1	08-08-2007	1	2	1	1	1	6	1	08-02-2008	2	0	17			IMR			20-01-2014
43	233	53	3	1	21-07-2007	1	2	1	1	1	6	1	18-02-2008	2	0	20			IMR			23-01-2014
44	235	40	4	1	09-08-2007	1	3	1	1	1	6	3	07-04-2008	0	0	17						25-04-2013

45	238	50	3	1	15-08-2007	1	2	1	1	1	6	1	06-02-2008	1	0	15						07-11-2013
46	240	49	2	2	22-12-2007	1	3	2		1	3	1	10-01-2008	1	1	11	1	3	IMR			17-03-2014
47	243	41	3	1	29-08-2007	1	3	1	1	1	6	1	28-01-2008	2	1	17			IMR			09-08-2013
48	263	47	3	1	18-09-2007	1	2	1	2	1	6	1	07-03-2008	0	1	6						09-08-2013
49	272	48	4	2	20-09-2007	1	3	1	1	1	6	3	22-02-2008	0	0	10						17-03-2014
50	275	38	4	1	24-09-2007	1	2	1	1	1	6	2	14-01-2008	3	1	10						23-12-2013
51	285	45	3	1	01-10-2007	1	2	1	1	1	6	1	07-03-2008	1	0	15						13-03-2013
52	286	38	4	2	01-10-2007	1	3	2	2	1	6	2	13-03-2008	0	0	12			IMR			01-08-2013
53	287	31	3	2	01-10-2007	1	3	1	2	1	4	1	29-01-2008	0	0	10	1	2				07-01-2013
54	289	22	3	1	06-10-2007	1	2	2	2	4	6	1	24-03-2008	0	0	13			IMR			24-07-2013
55	293	50	3	1	10-10-2007	1	3	1	1	1	6	1	16-04-2008	0	0	11			IMR			02-05-2013
56	296	47	3	1	11-10-2007	1	3	1	2	1	6	1	31-03-2008	0	0	24			IMR			29-01-2014
57	297	37	3	2	02-10-2007	1	3			1	6	1	02-05-2008	0	0	13						30-12-2013
58	301	47	3	1	07-04-2007	1	3	1	2	1	6	1	26-10-2007	2	0	17			IMR	07-02-2009	11-03-2009	11-03-2009
59	312	46	4	1	18-04-2007	1	2	2	1	1	6	1	14-09-2007	1	1	11			IMR			11-06-2013
60	316	50	3	1	21-04-2007	3	2	1	1	3	6	1	14-09-2007	1	0	11			IMR			23-12-2013
61	317	50	3	2	10-04-2007	1	3	2	2	1	6	1	03-12-2007	0	0	10			IMR			17-03-2014
62	318	33	4	1	13-04-2007	1	3	1	1	1	6	1	02-10-2007	1	1	7			IMR	29-09-2009	17-07-2010	17-07-2010
63	321	70	4	1	12-04-2007	1	2	2	1	3	6	1	07-09-2007	0	0	8			IMR	16-12-2010		16-12-2010
64	322	40	3	1	03-04-2007	1	3	2	2	1	3	1	17-07-2007	1	0	11	3	3	IMR	14-04-2009	18-09-2011	14-04-2009
65	323	29	3	1	17-04-2007	1	2	1	2	1	6	1	28-09-2007	1	0	12			IMR	10-12-2008	04-09-2009	04-09-2009
66	326	40	4	1	16-04-2007	1	2	1	1	1	6	1	30-10-2007	1	1	10			IMR			25-11-2013
67	334	35	4	1	24-04-2007	1	3	2	2	1	6	1	17-09-2007	0	0	16			IMR			06-09-2013
68	336	31	3	1	17-04-2007	1	3	2	2	1	6	1	21-09-2007	1	0	15			IMR			16-05-2013
69	339	60	4	1	25-04-2007	1		1	1	3	6	1	18-09-2007	1	0	9			IMR			06-12-2013
70	342	48	3	1	17-04-2007	1	2	2	2	1	6	1	18-09-2007	1	0	15			IMR			10-11-2012
71	355	43	3	1	03-05-2007	1	2	2	2	3	6	1	31-08-2007	0	0	15			IMR			09-07-2013
72	356	55	3	2	01-05-2007	1	2	1	2	1	6	1	17-09-2007	2	2	6			IMR			19-09-2013
73	366	53	3	2	21-04-2007	1	2	2	2	1	6	1	04-02-2008	0	0	13			IMR			29-08-2013
74	369	42	3	1	10-05-2007	1	3	2	2	1	6	1	12-10-2007	0	0	10			IMR			14-02-2014
75	374	41	2	2	11-05-2007	1	2	2	1	2	6	1	23-10-2007	0	1	10			IMR	04-03-2008		
76	378	27	3	2	10-05-2007	1	3	1	1	2	6	1	13-11-2007	0	1	15			IMR	23-08-2008	03-01-2009	03-01-2009
77	383	50	3	1	01-05-2007	1	3	2	2	1	4	1	10-08-2007	0	0	10				08-09-2007	25-08-2009	25-08-2009
78	389	50	3	1	15-05-2007	1	2	1	1	3	4	1	21-08-2007	1	0	8	3	2	IMR			16-05-2013
79	392	35	3	1	17-05-2007	1	2	2	2	2	4	1	27-08-2007	1	1	10	2	2	IMR			03-01-2013
80	400	47	3	1	21-05-2007	1	2	1	1	3	4	1	13-09-2007	1	2	6	3	2	IMR			14-04-2013
81	403	45	3	2	14-05-2007	1	3	2	2	1	6	1	26-11-2007	0	6	7			IMR	12-01-2008	15-12-2008	15-12-2008
82	415	35	4	1	24-05-2007	1	3	2	2	1	4	1	04-09-2007	2	2	13			IMR		10-12-2007	10-12-2007
83	455	53	4	1	15-06-2007	1	3	2	2	1	6	1	29-11-2007	0	0	9			IMR			15-05-2013
84	464	45	3	1	13-06-2007	1	3	2	2	1	6	1	11-12-2007	2	0	8			IMR			04-06-2013
85	470	65	4	2	27-06-2007	1	2	1	1	3	4	1	16-10-2007	2	6	13	3	2	IMR			17-05-2013
86	471	38	3	1	20-06-2007	1	2	1	1	3	5	1	02-10-2007	2	0	16	3	1	IMR			06-03-2013
87	480	44	3	1	23-06-2007	1	3	2	1	1	6	1	10-12-2007	1	0	5			IMR			04-04-2013
88	493	56	3	1	29-06-2007	4	3	1	1	3	6	1	20-11-2007	2	0	24			IMR			23-01-2014
89	495	38	3	1	27-06-2007	1	2	1	1	1	6	1	03-12-2007	1	0	22			IMR	29-06-2010		05-11-2013

90	500	55	4	1	27-06-2007	1	3	2	2	1	6	1	23-11-2007	0	0	9			IMR			24-12-2013
91	515	58	3	1	18-07-2007	1	2	1	1	1	5	1	06-11-2007	2	0	11	1	1	IMR			17-05-2013
92	521	32	4	1	03-07-2007	1	3	1	1	1	6	1	28-12-2007	1	0	12			IMR			03-07-2013
93	524	40	3	1	07-07-2007	1	2	1	1	1	6	1	24-12-2007	3	0	5						20-11-2013
94	528	40	4	2	18-07-2007	1	3	2	2	1	6	5								26-08-2008	26-08-2008	26-08-2008
95	531	58	3	1	13-07-2007	1	2	1	2	2	6	3	24-12-2007	0	0	6			IMR			17-05-2013
96	539	50	3	1	21-07-2007	1	2	1	1	2	6	1	13-07-2010	0	0	11						22-10-2013
97	541	45	3	2	09-07-2007	1	2	1	1	1	6	1	04-12-2007	1	0	14						21-01-2014
98	545	45	4	2	18-07-2007	1	2	1	1	1	6	1	29-11-2009	0	0	7			IMR			10-07-2013
99	550	67	3	1	25-07-2007	1	3	1	1	3	6	1	07-12-2007	1	0	15			IMR			26-06-2013
100	552	46	3	1	26-07-2007	1	2	1	1	1	6	1	16-11-2007	2	4	12			IMR			09-11-2013
101	558	35	4	2	01-08-2007	1	2	1	1	2	6	2	11-12-2007	0	2	4			IMR	28-09-2009	04-09-2010	04-09-2010
102	568	60	4	1	28-07-2007	1	2	1		3	3	1	19-11-2007	0	0	8	3	3	IMR			12-05-2009
103	572	59	3	1	26-07-2007	1	2	1	1	2	4	1	12-11-2007	1	0	10	2	3	IMR			20-03-2013
104	574	38	4	2	28-07-2007	1	3	1	1	1	6	2	29-02-2008	2	4	11				04-02-2011	08-02-2012	08-02-2012
105	575	42	3	1	01-08-2007	1	2	1	2	1	6	1	21-12-2007	1	0	18			IMR	07-06-2010		28-01-2011
106	576	41	3	1	04-08-2007	1	3	2	2	1	6	1	23-11-2007	1	0	25			IMR			02-12-2013
107	583	45	3	1	04-08-2007	1	3	1	1	1	6	3	14-12-2007	0	0	21			IMR	16-07-2008	04-12-2008	04-12-2008
108	588	44	3	1	09-08-2007	1	2	1	1	1	6	1	31-12-2007	1	3	10			IMR			13-02-2013
109	591	26	2	2	16-08-2007	1	3	1	1	1	6	1	25-01-2008	0	0	9						22-11-2013
110	594	40	3	1	04-09-2007	1	3	1	1	2	5	1	11-02-2008	1	0	18						17-05-2013
111	615	50	4	1	15-10-2007	1	2	1	1	1	4	1	17-06-2008	1	0	16					11-12-2010	11-12-2010
112	616	52	4	1	22-10-2007	1	2	1	2	1	6	1	06-03-2008	1	0	10			IMR	28-06-2010		28-06-2010
113	622	45	3	1	23-10-2007	1	3	1	1	2	6	1	18-04-2008	1	11	15			IMR	16-09-2008	07-10-2008	07-10-2008
114	624	50	3	1	09-10-2007	1	2	1	1	2	6	1	13-06-2008	2	0	10			IMR			22-10-2013
115	629	55	3	1	22-10-2007	3		1	2	2	6	1	18-03-2008	2	0	12			IMR			04-01-2014
116	639	67	4	1	13-10-2007	4	3	1	1	2	6	3	02-05-2008	0	0	12			IMR			18-12-2013
117	642	54	4	1	15-10-2007	1				1	6	2	02-04-2008	3	0	12						15-06-2010
118	654	48	4	2	06-11-2007	1	2	2	2	2	6	1	13-06-2008	2	1	17			IMR	19-07-2009	04-09-2010	04-09-2010
119	673	50	3	1	10-11-2007	1	3	2	2	1	6	1	25-04-2008	0	0	8			IMR			17-09-2013
120	674	44	3	2	16-11-2007	1	2	1	1	2	6	4	14-04-2008	1	10	13			IMR	19-10-2009	16-11-2012	16-11-2012
121	675	46	3	1	06-11-2007	1	3	2	2	1	6	1	02-04-2008	0	0	9			IMR			20-08-2013
122	677	47	3	1	22-11-2007	1	3	1	1	1	6	1	09-05-2008	0	0	11			IMR	11-08-2011	05-01-2012	05-01-2012
123	679	50	3	1	17-11-2007	1	2	1	2	2	6	1	04-04-2008	2	0	15			IMR	01-04-2010	05-01-2011	05-01-2011
124	684	42	3	1	30-11-2007	1	3	2	2	1	4	1	29-02-2008	2	0	7			IMR			13-08-2013
125	686	43	3	2	10-11-2007	1	2	2	2	2	6	1	22-07-2008	1	8	13			IMR	26-06-2012		08-11-2013
126	691	52	3	2	21-11-2007	1	3	2	2	1	6	1	02-05-2008	0	0	15			IMR	29-10-2008	03-09-2009	03-09-2009
127	696	47	3	1	28-11-2007	1	3	2	2	3	6	1	12-05-2008	0	0	13						15-07-2013
128	701	58	4	1	22-11-2007	1	2	1	1	3	6	1	12-05-2008	1	1	8			IMR	27-06-2011	17-08-2012	17-08-2012

129	702	62	2	2	04-12-2007	1	2	1	1	3	6	1	13-05-2008	1	5	12			IMR			27-11-2010
130	702	69	3	1	27-11-2007	1	2	2	2	2	6	1	23-05-2008	0	0	10			IMR	11-05-2012		05-02-2014
131	714	61	3	1	08-12-2007	1	3	1	1	3	4	1	02-04-2008	0	0	11						15-05-2013
132	715	37	3	2	11-12-2007	1	3	1	2	1	6	1	07-05-2008	2	1	16			IMR	08-11-2008	19-12-2008	19-12-2008
133	719	45	4	1	11-12-2007	1	2	2	2	1	3	1	29-04-2008	0	0	16			IMR			19-05-2013
134	737	50	4	1	25-12-2007	1	2	2	1	1	6	1	01-04-2008	0	0	7			IMR	01-08-2008	15-01-2009	15-01-2009
135	738	40	2	2	05-12-2007	1	3	2	2	1	6	1	09-06-2008	0	3	14			IMR	24-06-2010	11-12-2011	11-12-2011